A SOLID-PHASE RADIOIMMUNOASSAY FOR PLASMA PROGESTERONE

by

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ABBREVIATIONS

Trivial names of the steroids used:

Trivial Name	Systematic Name
Progesterone	4-pregnene-3,20-dione
6 β Hydroxyprogesterone	4-pregnene-6 β-ol-3,20-dione
6 β -Hydroxyprogesterone hemisuccinate	4-pregnene-6 β-ol-3,20-dione hemisuccinate
6∝ -Hydroxyprogesterone	4-pregnene-6∝ -ol-3,20-dione
7∝ -Hydroxyprogesterone	4-pregnene-7∝ -ol-3,20-dione
11∝ -Hydroxyprogesterone	4-pregnene-11∝ -ol-3,20-dione
11∝ -Hydroxyprogesterone hemisuccinate	4-pregnene-11∝ -ol-3,20-dione hemisuccinate
11 β -Hydroxyprogesterone	4-pregnene-11 β-ol-3,20-dione
17-Hydroxyprogesterone	4-pregnene-17∝ -ol-3,20-dione
11-Ketoprogesterone	4-pregnene-3,11,20-trione
Pregnenolone	5-pregnene-3 β-ol-20-one (3β -Hydroxypregn-5-en-20-one)
17-Hydroxypregnenolone	5-pregnene-3 β,17∝ -diol-20-one (3 β,17∝ -dihydroxy-5-pregnene-20-one)
Testosterone	4-androsten-17 β ol-3 one (17 β -hydroxy-4-androsten-3-one)
Oestrone	1,3,5(10)-oestratrien-3-ol-17-one
Oestradiol	1,3,5(10)-oestratrien-3,17 β -diol
Oestriol	1,3,5(10)-oestratrien-3,16∝ ,17β-triol
Dehydroepiandrosterone (DHA)	5-androsten-3 β-ol-17-one (3 β-hydroxyandrost-5-en-17-one)
Cortisol	4-pregnen-11 β,17∝ ,21-triol-3,20-dione

Trivial Name	Systematic Name
Corticosterone	4-pregnen-11β,21-diol-3,20-dione
Cortisone	4-pregnen-17α,21-diol-3,11,20-trione
Aldosterone	4-pregnen-11β,21-diol-3,18,20-trione (4-pregnen-11β,21-diol-3,20-dione-18-al)
11-deoxycortisol (compound S)	4-pregnen-17α,21-diol-3,20-dione
11-deoxycorticosterone (DOC)	4-pregnen-21-ol-3,20-dione
Cholesterol	5-cholesten-3β-ol

Proprietary Name	Systematic Name
Fluorinef	9α-Fluro-11β,17α-dihydroxy,21-acetoxy pregnene-3,20-dione
Medroxy progesterone	17α-Hydroxy-6α-methylpregnene-3,20-dione
Mesterenol	17α-Ethynyl-3-methoxyoestra-1,3,5(10)-trien-17β-ol
Chlormadinone	6-Chloro-17α-hydroxypregna-4,6-diene-3,20-dione
Indomethacin	1-(4-Chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid
Cyanoketone	2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one
Oxymetholone	17β-Hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one
Dexamethasone	9α-Fluro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione
Fluoxymesterone	9α-Fluro-11β,17β-dihydroxy-17α-methylandrost-4-en-3-one

Abbreviations

g	gram
mg	$g \times 10^{-3}$
µg	$g \times 10^{-6}$
ng	$g \times 10^{-9}$
pg	$g \times 10^{-12}$
I.U.	International Units
vol.	volume
l.	litre
µl	microlitre
cm.	centimetre
Ci.	curie
mCi	millicurie (curie $\times 10^{-3}$)
µCi	microcurie (curie $\times 10^{-6}$)
cpm.	counts per minute
rpm.	revolutions per minute
h	hour
min	minute
S.D.	standard deviation
BSA	bovine serum albumin
NRS	non immune rabbit serum
DARS	donkey anti rabbit serum

FSH	follicle stimulating hormone
PMS	pregnant mare serum
LH	luteinizing hormone
HCG	human chorionic gonadotropin
TSH	thyroid stimulating hormone
ACTH	adrenocorticotrophic hormone
AMP	adrenosine mono phosphate
I.V.	Intra Venous
I.M.	intra-muscular
G.L.C.	gas-liquid chromatography
T.L.C.	thin-layer chromatography
U.V.	ultra violet spectroscopy
nm	nano meter
RIA	Radioimmunoassay

CONTENTS

SUMMARY

This thesis describes in detail the methodology involved in developing a solid phase radioimmunoassay for progesterone in human plasma as well as some applications.

The introduction reviews progesterone as a hormone involved in reproduction and describes its role in the normal menstrual cycle, in pregnancy and traces briefly the important steps in the history of its measurement in the human blood.

Hemisuccinate derivatives of 11α-hydroxyprogesterone and 6β-hydroxyprogesterone have been prepared and conjugated to bovine serum albumin by the mixed anhydride reaction. Antisera in 8 of the 12 rabbits gave usable dilutions of 1/50000 or more. The average affinity constants of the three selected antisera to 11α-hydroxyprogesterone-BSA are 3.15×10^9, 3.77×10^9 and 3.92×10^9 1/M and those of three selected antisera to 6β-hydroxyprogesterone-BSA are 2.92×10^9, 3.55×10^9 and 3.92×10^9 1/M. Maximum assay sensitivities with

antisera were generally obtained after 3-7 months from primary immunization and remained unchanged thereafter following further booster injections.

Extensive studies on the specificity of the antisera have been carried out and discussed. Cross reaction studies of 8 compounds with selected antisera to 11α-hydroxyprogesterone-BSA showed little alteration in their specificities following booster injections over long periods.

The routine procedure is based upon the use of an antiserum to 11α-hydroxyprogesterone-BSA which is covalently linked to microcrystalline cellulose, the double antibody method being used as a reference system. In the extraction procedure ethanol is first added to the plasma (10 Vol. plasma + 1 Vol. ethanol) after which a single extraction with light petroleum yields a constant recovery 92.4 $\pm$ 1.2 (S.D.) % of added [^{3}H]progesterone and obviates the need for tracer recoveries on each sample being assayed. The effect of the solvent residues on the assay has been investigated and distortions of the response curve due to this have been almost eliminated.

A 200µl plasma sample is sufficient for the measurement of progesterone at all stages of the menstrual cycle. Other reliability criteria, accuracy precision, specificity and sensitivity have been shown to be satisfactorily met.

The method has been applied for assaying the hormone in the urine of the normally menstruating women.

The behaviour of unextracted plasma in the assay has been briefly investigated because the specificity of the antisera to known steroids appeared to permit such assays at least for the limited but important purpose of monitoring luteal function. However, grossly high and unacceptable values were obtained when such assays were performed.

The following physiological situations have been studied:

Progesterone plasma levels have been measured in daily samples throughout a single menstrual cycle from five women. The first detectable rise of progesterone is recorded only after the start of the midcycle rise of luteinizing hormone.

Effect /

Effect of ACTH stimulation and dexamethasone suppression on progesterone levels has been studied in males. A four-fold but slow rise over 6 hours in response to ACTH suggests a slow rate of synthesis in the adrenals. Progesterone was still detectable in plasma from normal men during dexamethazone suppression.

Studies from frequent blood sampling over a 6 h period in three males showed no evidence of a regular pattern of episodic secretion of progesterone. No relationship was observed between the secretory patterns of progesterone and those of luteinizing hormone and testosterone which were measured in the same blood samples.

(The method for measuring progesterone as described in this thesis has been published as "A solid-phase Radioimmunoassay for Plasma Progesterone"; Dighe K.K. and W.M. Hunter (1974), Biochem. J. 143, 219 - 231).

I N T R O D U C T I O N

CHAPTER 1

P R O G E S T E R O N E

CHAPTER 1

PROGESTERONE

General Introduction

The corpus luteum of ovary finds its first recorded mention in 1672 (de Graff, 1672). Its function as an organ of internal secretion in the maintenance of pregnancy was proposed by Prenent (1898). Continued interest in the organ culminated in the publication of the first significant evidence of the biological importance of its secretion when it was demonstrated that corpus luteum extracts from the sow were able to maintain pregnancy in castrated rabbits (Corner and Allen, 1929). Improved techniques led to the isolation of the hormone responsible for this effect from corpora lutea of several species. Elucidation of its structure followed and the name progesterone was proposed (Butendant, et al., 1934).

Progesterone, pregn-4-ene-3, 20-dione has an empirical formula for $C_{21}H_{30}O_2$ and molecular weight of 314.4. It is the least polar of all the naturally occurring steroids, its solubility in water being low, while it dissolves readily in organic solvents. It is synthesized from cholesterol, pregnenolone - their sulphates, and from acetate, and is one of the key intermediates in the biosynthesis of steroids on the Δ^4 - pathway in the ovary, testis and adrenals.

Protein binding of progesterone in plasma

Progesterone is transported in blood bound to certain plasma proteins. The known proteins are cortico steroid binding globulin (CBG), albumin and ∝1- acid glycoprotein. The affinity of this binding is dictated by the affinity constant (k-value) of the binding reaction. CBG binds progesterone with high affinity, of the order of 10^{7-8} l/m, but its concentration in plasma is only about 3.8 mg/l. The affinities of albumin or ∝1 - acid glycoprotein, to which progesterone also binds, are of a lower order (10^{4-5} l/m) but their plasma concentrations are higher (750 mg/l for ∝ 1- acid glycoprotein, and 50 times more for albumin). Yannone, et al., (1969), have calculated the total amount of protein bound progesterone to be 91% in pregnant and 92% in women in the normal menstrual cycles. The albumin-bound and possibly also the ∝ 1- acid glycoprotein-bound fraction, in pregnant and the non-pregnant subject are calculated as 50% and 70% respectively, while the CBG-bound fraction in the same categories were calculated as 40% and 22% respectively (Rosenthal, et al., 1969).

In vitro experiments protein-bound progesterone has been shown to be inactive in exhibiting its specific hormonal activity (Westphal and Forbes, 1963; Hoffman, et al., 1969), which suggests that protein binding of progesterone in vivo may be a mechanism by which progesterone makes itself less available to the target tissues. The binding of progesterone

to the proteins is by non-covalent and reversible bonds so that protein-bound progesterone can be made available by dissociation to those tissues whose affinities for progesterone exceed those of the binding proteins. It has been shown that albumin and/or α_1 glycoprotein-bound progesterone is more readily extracted (irreversibly metabolized) by the liver than the portion bound to CBG which is protected from such metabolism (Rosenthal, et al., 1969). Binding of progesterone to proteins of high affinity with low capacity, and to proteins of low affinity with high capacity can therefore have major effects on its metabolism and hormonal activity.

The presence of a specific progesterone-binding plasma protein in the pregnant guinea pig has been reported (Diamond et al., 1969; Milgrom, et al., 1970). Binding of progesterone in these animals is raised 100-fold as compared to non-pregnant animals, whereas cortisol-binding is raised only by a factor of 4.

Clearance and metabolism in man

The plasma half-life of progesterone has been determined by various procedures: (a) following expulsion of the placenta it was found to have a half-life of 5 min. (Short and Eton, 1959), (b) following injections and infusions of labelled and unlabelled progesterone in two non-pregnant subjects the half-life was

calculated as 25 and 29 min. (Thijssen and Zander, 1966; Little et al., 1966). (c) following the simultaneous removal of the foeto-placental unit and corpus luteum in the first trimester of pregnancy the disappearance of progesterone was observed to take place in two phases. The half-life from the disappearance in the first phase was calculated as 6 min. and from the second phase disappearance as 43 min. (Fylling, 1971). The two-phase disappearance has been explained as follows: progesterone is rapidly cleared upon entering the vascular compartment. Clearance is then slowed as it comes in contact and equilibrates with the tissues (outer pool) from where it then slowly returns to the blood.

Thus the available evidence indicates that progesterone is cleared rapidly from the blood. It is not stored in the corpus luteum as the turnover time, that is, the time taken for the corpus luteum to release its content of progesterone into the blood, is only 10 min (Short et al., 1963). The amount of progesterone in the term placentas has been calculated to be 750µg (Zander and von Münstermann, 1956). Since this amount of progesterone is equivalent to the placental secretion for 5 min. (250 mg/day), this finding also suggests that progesterone is not stored in the placenta (Van der Molen and Aakvaag, 1967).

Studies on the metabolic clearance rates of progesterone have shown that about half the progesterone given by I.V.infusion

is metabolized by organs other than liver (Little et al., 1966). Changes in the equilibrium and the extent to which ratios of free progesterone to that which is bound to the different plasma proteins change when blood passes through the various receptor organs are not fully understood. The metabolic clearance rate of progesterone is the same in males, ovariectomised women and pregnancy, but is about 25% greater in menstruating women in whom however, no difference in metabolic clearance rates is observed between follicular and luteal phase (Lin et al., 1972).

Progesterone is usually metabolized by reduction, hydroxylation and conjugation, but the products vary in specific tissues. The liver metabolizes progesterone mainly to ring A-5 β-pregnane derivitives and hydroxylation products (Dorfman and Ungar, 1965). The target tissues (endometrium and myometrium) metabolize progesterone mainly to ring A-5α - pregnane derivitives and hydroxylation products (Bryson and Sweat. 1967, 1969).

Physiologic and Biological role

The main physiological effects of progesterone have been described (O'Malley, 1969) as follows:

(a) Preparation of uterine endometrial cells so that favourable conditions are present for the receipt and implantation of the embryo.

(b) Exertion of a "blocking effect" to reduce the excitation of uterine muscles resulting in stoppage of their rhythmic

contraction - excitation activity (Csapo, 1961). This aids in the retention of the embryo in the uterus.

(c) Stimulation of the alveolar development of breast tissue and so preparing it for lactation.

The present evidence suggests that metabolites of progesterone, e.g. 5α-pregnane - 3,20 - dione and 20 - hydroxypregn-4-en-3-one are less active than progesterone itself. Experiments in rats showed greater enzyme activity of Δ^4-5 - reductase, 3α- hydroxysteroid dehydrogenase and 20α- hydroxy-steroid dehydrogenase in the oestrogen dominated phase (oestrus) reflecting greater progesterone metabolism than in pseudopregnancy or pregnancy (Wiest, 1969). The concentrations of progesterone in rat myometrium were higher than in peripheral plasma in early pregnancy, while no differences in the levels of 20α-hydroxypregn-4-ene-3-one were observed between the two. This suggests a longer half-life of progesterone in the myometrium (Wiest, 1969). 20α- Hydroxypregn-4-ene-3-one is ineffective in supporting decidual reaction, during implantation and in maintaining or prolonging the pregnancy (Wiest, 1969). 5α- Pregnane 3,20-dione when administered exogenously in rats showed weaker biological activity than progesterone (Armstrong, 1970). From the available evidence it therefore appears that the biological activity of progesterone resides in the molecule itself (Wiest, 1969).

Role in Male Physiology:

The available evidence does not support the view that there is a physiological role for progesterone production from the testis. In males, circulating progesterone is mainly adrenal in origin. Blood production rates in males and ovariectomised women are not significantly different (Little et al 1966).

Biosynthetic evidence leads to a similar conclusion. Progesterone is not found concentrated in testicular tissue. It is also shown to inhibit the rate of formation of androgen because of its inhibitory activity on the enzyme system - the C-17-20-lyase responsible for side chain cleavage (Huseby et al 1961). *In vitro* evidence in testis suggests that the Δ^4 pathway which includes progesterone as an intermediate is of minor importance in the synthesis of androgens (Yanihara and Troen 1972).

Failure of plasma progesterone levels to change in response to HCG stimulation or fluximestarone supression (Strott et al., 1969) provides further evidence against any significant contribution of the testis to circulating progesterone levels.

Role in Adrenal Physiology:

Hechter and Pincus (1954) first suggested that progesterone was the key intermediate in the synthesis of cortisol from their invitro studies on the adrenal tissue. This notion was however disproved by the invitro studies of Weliky and Engel (1963) on hyperplastic human adrenal cortical tissue when they showed that radioactive pregnenolone was converted to cortisol independantly through 17-hydroxy pregnenolone, 17-hydroxy progesterone and deoxy-cortisol while no conversion to progesterone was observed. Deshpande et al. (1970) injected radioactive pregnenolone, 17-hydroxy pregnenolone and progesterone into the adrenal artery of patients with advanced breast cancer and from the isolation of steroids in the adrenal vein demonstrated that cortisol was mainly synthesized from Δ^5 steroids rather than from progesterone. This was confirmed using larger amounts of endogenous Δ^5 -steroids in tissue pools. Infusions of ACTH over a 3 h[illegible] period in normal men were found to produce a 4-6-fold response in the concentration of Δ^5 steroids accompanied by only a 2-fold response in the concentration of progesterone and 17-hydroxy progesterone (Bermudez and Lipsett 1972). This suggests progesterone involvement only in a minor way in the synthesis of cortisol, at least under conditions of stimulation or stress.

A continuous basal secretion of progesterone by the adrenal gland has yet to be demonstrated. Progesterone has been identified in adrenal vencus effluent of postmenopausal women after stimulation with ACTH (Short 1960). That plasma progesterone rises in response to ACTH and not to HCG stimulation (Strott et al., 1969a) strongly suggests that the increment is secreted by the adrenal glands although this does not prove its basal secretion by the adrenal gland.

Role in Female Reproduction:

When the concentration of a hormone in the venous blood of an endocrine organ is greater than that in the arterial blood supplying that organ or in the peripheral blood, the hormone is considered a secretory product of that endocrine organ (Eikness and Hall 1965). By this definition progesterone clearly qualifies as a secretory product of the ovary (Mikhail 1970, Lloyd et al., 1971).

Of the main steroid-producing functional units of the ovary, the follicle, corpus luteum and the stromal or interstitial tissue; the stromal tissue is the least significant source of ovarian progesterone. This has been

shown in invitro experiments with stromal tissues from normal human ovarian sources (Rice and Savard 1966) and also in in vivo systems when the concentration of progesterone in the ovarian vein on the side of the contralateral ovary was shown to be significantly lower than that from the ovary containing the ripening follicle (Mikhail 1970, Lloyd et al., 1971).

Graafian Follicle:

Two cell types constitute the human Graafian follicle - the granulosa and theca interna cells. The former envelopes the oocyte and developes several layers with the growth of the follicle. The granulosa cells are devoid of blood vessels whereas the theca interna cells (the outer layer being theca externa) are well supplied with a plexus of blood capillaries and lymphatics. Biosynthetically, in in vitro experiments, theca interna cells have been shown to utilise the Δ^5- pathway more efficiently than the granulosa cells which again in in vitro studies have been shown to synthesize progesterone as the major product (Ryan and Petro 1966). The synthetic capacity of granulosa cells to secrete progesterone at different stages during the follicular phase was also shown by the in vitro tissue culture experiments using these cells (Channing 1970).

Although this in vitro biochemical evidence demonstrates

the synthetic competence of granulosa cells to synthesize progesterone, the amount secreted by the follicle *in vivo* is small as judged by the peripheral plasma progesterone concentrations in the follicular phase of the cycle. A significant portion of the follicular-phase progesterone is considered to be adrenal in origin. Progesterone levels in the follicular-phase of normally menstruating women increased four-fold in response to ACTH stimulation (Strott et al., 1969a). However, significant amounts of progesterone were measured in the follicular phase of a bilaterally adrenalectomized woman (Abraham et al., 1973a) maintained on replacement therapy with fluorinef (0.05 mg/day) and cortisol acetate (40 mg/day).

These findings together pose the question as to why the granulosa cells which synthesize appreciable amounts of progesterone *in vitro* actually secrete only very small amounts *in vivo*. One explanation was based on an observation that removal of the oocyte from mature follicles of oestrus rabbits stimulated progesterone secretion by the granulosa cells (El fouli et al., 1970). This suggested the presence of a luteinizing inhibiting factor associated with the presence of the oocyte (Armstrong 1970a). The progesterone secretion by the granulosa cells of mature

follicles in culture medium (Channing 1970) where they were removed from proximity to the oocyte was cited as additional evidence for the presence of the luteinizing inhibiting factor. As the luteinization of granulosa cells in culture medium does not involve a surge of LH and FSH it was suggested that the function of such a surge in a normal cycle was to suppress the ovum-induced signal to luteinization (Schwartz and McCormack 1972). However there is no direct evidence for the presence of a luteinizing-inhibiting factor and whether replacing the oocyte in the follicle would inhibit the secretion of progesterone is not known (Baird 1973).

An alternative explanation for the low progesterone levels in the follicular phase is based on the lack of adequate oxygen and food supply required for full steroidogenesis by the granulosa cells. The granulosa cells are devoid of blood vessels and the synthesized progesterone may not reach the vascular system other than by diffusion. For the same reason the required nutrients and precusers for steroid synthesis may reach these cells in sub-optimal amounts (Short 1964). It has recently been shown that the partial pressure of oxygen in the follicle is low until the midcycle LH surge when secretion of progesterone commences (Fraser et al., 1973). Present

evidence therefore suggests that the partial pressure of oxygen in the follicle is altered under the influence of the midcycle LH surge and that this is associated with the stimulation of steroidogenesis including progesterone synthesis.

Role for Progesterone during Mid-Cycle:

Under experimental conditions both progesterone and oestradiol have been found capable of inducing a surge of LH and/or FSH of the magnitude similar to that of the midcycle peak of gonadotropins. However there is overwhelming evidence in favour of oestradiol rather than progesterone as the natural trigger of the midcycle gonadotropin surge in the menstrual cycle.

In earlier studies on normally menstruating women a urinary oestrogen peak was shown to occur coinciding with or preceding the midcycle peak of gonadotropins by 2 or one days whereas the rise in urinary pregnanediol levels occurred only 1 - 4 days after the oestrogen peak (Brown 1962, and Matthew Burger et al 1968, Goebelsmann et al.,1969). Later studies on the normal menstrual cycle, in which plasma oestradiol and LH levels were measured simultaneously by sensitive

techniques, also showed a rise and fall of oestradiol before the LH peak (Corker et al., 1969). The ability of oestradiol to induce an LH peak has been shown by giving multiple subcutaneous injections or single intramuscular injection of oestradiol benzoate to rhesus monkeys in which elevated levels of plasma oestradiol sustained for more than 12 h induced the LH peak resembling the midcycle peak after 24 - 48 h (Knobil et al., 1972). Administration of exogenous oestrogens for 1 - 3 days during the mid-follicular phase (days 7-9) resulted in a surge of gonadotropins, after 12 - 24 h, similar to those occurring spontaneously in the midcycle. Vandewiele et al. (1970) observed that in some infertile women who were being treated with gonadotropins, ovulation occurred in the FSH phase of the treatment even before the administration of HCG which was given to induce ovulation. This ovulation was preceded by an increase in urinary oestrogens without a concomitant rise in pregnanediol levels. Johansson and Wide (1969) in a detailed study on the relationship between the midcycle LH release and changes in plasma progesterone levels showed that the first detectable rise in progesterone concentration occurred on the day of the LH peak and therefore some 24 h after

the first detectable increase in plasma LH. Similar observations have been made by Vandewiele et al. (1970). All the evidence therefore implicates oestradiol as the primary trigger for the midcycle surge of gonadotropins rather than progesterone which shows no change in its concentration of a kind which could act as a signal for the release of LH.

However, progesterone has also been shown to induce LH release under certain circumstances. In a group of castrate and postmenopausal women in whom endogenous gonadotropin levels were suppressed by diethyl stilboestrol (15 mg/day) or ethynyl oestradiol (0.2 - 0.5 mg/day), progesterone (10 mg intra muscular) or medroxy progesterone (10 - 20 mg I.M.) was then given. This treatment induced an LH and FSH surge closely resembling the midcycle gonadotropin peak. Further, a second gonadotropin surge was not obtained on continuation of the progestogen treatment. These observations lead the authors to suggest progesterone as the natural trigger for the midcycle surge of gonadotropins although they did not measure the circulating progesterone levels in their studies (Odell and Swerdloff 1968). Progesterone-induced LH/FSH surges were also observed by Nillius and

Wide (1971). Their studies were carried out on 20 postmenopausal women given 10 µg of ethynyl oestradiol daily for four weeks before starting the progesterone treatment (10 mg or 100 mg I.M. in oil). By measuring the plasma progesterone levels achieved with this regimen they showed higher doses to be less effective than the 10 mg dose. They also showed that when progesterone treatment was given in the absence of pretreatment with oestrogens, no major changes in the serum concentrations of gonadotropins were observed. However, the magnitude of the LH surges they obtained in most subjects were not particularly impressive and it is arguable that these surges were the result of a positive feedback response to progesterone administration or were in fact the natural episodic pattern of LH release of high amplitude and slow pulse rate observed in the luteal phase in women (Santen and Bardin 1973) and during progesterone administration in rhesus monkey (Knobil et al. 1972). The studies of Leydendecker et al. (1972) were carried out on a group of women that included castrate eugonadal, postmenopausal women and those with primary amenorrhoea who were all receiving 60 µg of ethynyl oestradiol per day before the experiments. These workers showed that intramuscular injections of varying doses, 20,50,200 mg of microcrystalline progesterone were able to induce an LH

surge within 3 - 16 h [illegible]. Following an intramuscular injection of 20 mg of microcrystalline progesterone the circulating levels in plasma rose, within 90 - 120 min[illegible], to 1-2 ng/ml, a concentration that is similar to that found on the day of the LH peak in the normal cycles. The injections at higher doses (50 or 200 mg) of progesterone produced circulating concentrations similar to the luteal phase levels. These experiments suggest that a progesterone-induced positive feedback effect is achieved once a certain threshold concentration of progesterone in plasma is reached. These experiments and the previous observations have led to the present concept of the role of progesterone in the positive feedback mechanism which gives rise to the midcycle LH surge. This is as follows: although progesterone does not trigger the midcycle gonadotropin peak (as its first detectable increase in concentration is seen only after the start of the LH surge), it may potentiate the oestradiol-initiated LH surge and so increase its magnitude (Leydendecker et al., 1972).

On the other hand progesterone, when present at certain inhibitory threshold levels, has also been shown to suppress the LH surge and thus inhibit ovulation. When progesterone was injected in a group of rhesus monkeys in doses of 0.5, 2 and 5 mg from the beginning of the menstrual

cycle, blocked equally at all dose levels both the rise of LH and ovulation as observed at laparatomy and by comparison with control animals (Spies and Niswender 1971). Failure of oestradiol to induce an LH surge in the presence of progesterone is shown by the experiments in the rhesus monkey (Knobil et al., 1972). Administration of oestradiol benzoate during the luteal phase of these animals failed to produce an LH surge when the progesterone concentrations were high (4 ng/ml). When the newly formed corpus luteum was removed and the concentrations of plasma progesterone had decreased to undetectable levels the oestradiol benzoate administered on the same day of the menstrual cycle produced a sharp LH peak. In the studies of Swerdloff and Odell (1969) on eugonadal women with normal cycles, there were multiple LH peaks in the oestrogenic phase during a regimen of oral sequential contraceptive pill (mesterenol, 80 µg for 15 days) but when a progestogen was administered (chlormadinone acetate 2 mg) only one sharp peak occurred. The inhibitory action of progesterone may be brought about by blocking the central nervous system as suggested by the experiments of Zeilmaker and Moll (1967). They were able to induce ovulation in 5 day cycling rats by electrochemical stimulation of the median eminence or the pre-optic area. However when 10 mg of progesterone was given on day-3

of the cycle ovulation was blocked and could be induced by the stimulation of median eminence. The role for the inhibitory effect of progesterone then lies in preventing a second surge of LH from being initiated by the luteal rise in oestradiol or progesterone itself (Leydendecker et al. 1972).

The positive feedback effect of progesterone thus offers a fail/safe mechanism whereby the oestradiol-initiated full surge of LH is ensured. The inhibitory effect of progesterone, by preventing further LH surges during the luteal phase allows only one ovulation to take place in the menstrual cycle.

Role in Ovulation:

The mechanism of follicular rupture which is followed by extrusion of the ovum is now generally considered to involve an increase in tension of the follicular wall.

Studies in the past have shown the involvement of progesterone in the process of ovulation. Some of these are based on its ability *in vitro* to increase the distensibility of the follicular wall. Progesterone, but not oestrogen, LH or cyclic AMP, was able to increase the distensibility

of the follicular wall as observed in culture experiments using follicular strips from the sow. Further, when this distensibility was blocked by the simultaneous addition of cyanoketone, an inhibitor of 3β - hydroxysteroid dehydrogenase involved in the conversion of pregnenolone to progesterone, only progesterone could reverse the blockade (Rondell 1970). Of the various steroid enzyme inhibitors used to block ovulation induced by LH in pregnant mare serum (PMS) treated rats, cyanoketone was the most effective (Lipner and Greep 1971).

Based on similar experiments the role of progesterone in the process of ovulation was explained as follows: The initiation of ovulatory process started with the midcycle LH surge finally resulting in the increased steroidogenesis. The main product, progesterone, then caused the stimulation of the collagenase-like enzyme destroying the collagen-like framework in the follicular wall. These developments caused the increase in distensibility of the follicular wall and finally its rupture (Rondell 1970, Schwartz and McCormack 1972).

More recent evidence, however, does not support a role for progesterone in the physical process of ovulation i.e. follicular rupture. In the rat there is a preovulatory retention of uterine fluid which reflects the inadequate

concentrations of progesterone necessary for cervical relaxation and hence uterine fluid release (Armstrong 1968). Ovulation in rats was blocked by giving the prostaglandin synthesis blocking agent, indomethacin. This blockade was not reversed by gonadotropin releasing factor although there was a loss of uterine fluid suggesting that LH was released by the pituitary and that this stimulated the production of progesterone necessary for cervical relaxation. Thus indomethacin does not appear to block ovulation by inhibiting ovarian steroidogenesis. Rather, it may block ovulation by preventing rupture of the follicle and release of the ovum (Behrman et al., 1972). Release of progesterone in the absence of ovulation (which again was blocked by indomethacin) was also observed in rats by Armstrong and Grinwich (1972). O'Grady et al. (1972) also were able to block LH-induced ovulation in rabbits by indomethacin although progesterone secretion was normal.

The Luteal-Phase:

The synthesis of progesterone by the corpus luteum is ascribed to the luteinised granulosa cells which together with the theca interna cells constitute the two cellular compartments of the corpus luteum. There is no evidence on the individual steroid secretion patterns of the two cell

types. In vitro studies in luteal slices have shown the presence of enzymes of both the Δ^4 and Δ^5 pathways and also that it is the Δ^4 pathway that is more active (Ryan 1963). That the Δ^5 pathway in the luteal phase is not more active is also suggested by the levels of Δ^5 -steroids (Pregnenolone, 17-hydroxy pregnenolone and Dehydroepiandrosterone), which as measured by radio immunoassay were not significantly different in the follicular and luteal phases (Abraham et al. 1973a).

Progesterone levels rise in response to the midcycle LH surge and reach peak levels about a week thereafter. Although the production rate of progesterone in the luteal-phase has been calculated to be 15 - 50 mg/day (Lin [illegible] et al., 1972) a dose of 5 mg/day was found sufficient to prevent bleeding following excision of the corpus luteum (Corner 1937). In another study an intramuscular injection of 12.5 mg/day was found adequate to maintain a secretory endometrium in the absence of endogenous progesterone production (Jones 1968). An intramuscular dose of 25 mg/day of progesterone was found to be the ideal dose in maintaining luteal phase plasma levels as measured by the competitive protein binding method (Nillius and Johansson 1971).

It has now been fairly well established that the corpus luteum does not function autonomously but that it requires low but continuous secretion of LH to maintain the normal luteal phase progesterone levels (Vandewiele et al., 1970). This is supported by experiments where administration of antisera to HCG (which cross reacted with LH) to rhesus monkey in their luteal phase resulted in reduced progesterone secretion and caused premature menstruation (Moudgal et al., 1972). Although it has a vital role in maintaining the corpus luteum in humans, LH seems unable to prolong the life span of the corpus luteum beyond a limited period. Even high daily doses (1600 I.U.) of LH given to hypophysectomised women were unable to maintain progesterone production beyond 22 days (Jewelewicz et al., 1973). The normal life-span of the corpus luteum in some animal species has been shown to be controlled by uterine luteolytic factors possibly prostaglandin F2α (Hillard 1973). But this does not appear to be the mechanism controlling corpus luteum regression in humans as hysterectomy has been shown not to affect normal luteal function (Beling et al., 1970). Also, luteal function was normal in women with congenital absence of uterus as judged by the timing and magnitude of urinary pregnanediol and oestrogen levels following the midcycle LH surge (Fraser et al., 1973b).

Attempts have been made to study the control of corpus luteum function by suppressing or blocking progesterone synthesis in the luteal-phase. One approach was by inhibiting the synthesis of progesterone directly by enzyme inhibitors. Cyanoketones and other androstane derivatives which block progesterone synthesis at the 3 β-hydroxy steroid dehydrogenase step also block adrenal synthesis of progesterone (Henzl and Segre 1970). Amino-gluthetimide (Salhanick et al., 1972) affects progesterone synthesis in both the ovary and the adrenals by blocking cytochrome P-450 activity which is necessary for the conversion of cholesterol to pregnenolone. A selective inhibition of ovarian synthesis of progesterone which does not interfere with its synthesis in the adrenal has not yet been accomplished (Jewelewicz et al., 1973). Prostaglandins infused during the luteal phase in doses sufficient to cause abortion in mid-trimester pregnancy failed to affect the normal luteal-phase (Jewelewicz et al., 1972).

In another approach for controlling corpus luteum function progesterone synthesis in the luteal phase has been shown to be suppressed in varying degrees by administering oestrogens (Gore et al., 1973), progestogens (Johansson 1971) and oxymethalone (Klaiber et al., 1973). Their actions are reversed by the exogenous administration of HCG suggesting

that these compounds suppress progesterone synthesis by the inhibition of LH secretion. However, oxymethalone also blocks 3 β-hydroxy steroid dehydrogenase and isomerase activity and its effects on corpus luteum function may therefore be complex.

Progesterone in Early human Pregnancy:

The fertilized ovum remains in the fallopian tube for 48 h before journeying to the uterus for implantation which is believed to begin on day 4 - 6 after entry into the uterus (Blandau 1961); the implantation being completed on day 12 (Henzl and Segre 1970). In response to implantation progesterone levels show a sharp increase above luteal phase levels from around day-8 onwards, at a time when they show a decreasing trend in a normal luteal phase (Johansson 1969; Knobil 1973). This increase in plasma progesterone concentration is believed to be due to luteotrophic stimulation by HCG. This is supported by the following evidence. Administration of HCG during the early or the mid-luteal phase was shown to increase the progesterone production 2-4 fold (Strott et al., 1969b). No qualitative differences in the stimulatory action of human LH or HCG upon progesterone production by corpus luteum were observed

when either of the two hormones were administered during the luteal-phase of women with normal menstrual cycles. Both LH and HCG increased the progesterone production 2-3 fold that of the control cycles and lengthened the life-span of luteal-phase for up to 8-9 days (Hanson et al., 1971). Ovulation has been induced by exogenous administration of HCG after pre treatment with HMG (to promote follicular development) in hypophysectomised (Gemzell 1965) and anovulatory women (Yoshimi et al., 1969); (ovulation so induced in these women also resulted in successful pregnancies).

However there are two principal objections (Short 1969) to HCG as the luteotrophic stimulus. One is the absence of HCG at the time of implantation on day 4-6 at a time when there is a significant increase in the synthesis of progesterone, HCG appearing in blood only on day-9 after the midcycle gonadotropin surge (Kosasa et al., 1973). The other objection is the inverse relationship in the circulating concentrations of HCG and progesterone. The levels of HCG continue to rise after implantation and reach a maximum between 5-9th week of gestation whereas progesterone concentration, although above luteal-phase levels, show a downward trend during this period (Yoshimi et al., 1969, Johansson and Wide

, 1969). This inverse relationship has been explained on the basis of refractoriness of the corpus luteum to the continuous and rising stimulus of HCG.

Generally the corpus luteum is essential for the foetus up to about 8-9 weeks of gestation. Removal of the corpus luteum before this period was shown to result in low progesterone levels and abortion. Removal after day 61 resulted in a slight transient decrease of progesterone levels followed by a significant rise without affecting the pregnancy, the rise pointing towards a shift of the site of progesterone production from the corpus luteum to the placenta (Csapo et al., 1972; Holmdahl et al., 1971). A similar shift of progesterone production to the placenta at about this time (days 50-60) is also evidenced by the divergent patterns of circulating levels of progesterone and 17-hydroxy progesterone. As the placenta lacks the enzyme 17α-hydroxylase the 17-hydroxy progesterone levels indicate the declining ovarian contribution and the rising progesterone levels the increasing placental contribution (Yoshimi et al., 1969). The essential contribution of the corpus luteum to circulating progesterone levels that are required for maintaining the pregnancy may in fact be even shorter than 50-60 days, as oophorectomy 35 days after ovulation has been shown not

to interrupt pregnancy (Tulsky and Koff 1957). The main role of the corpus luteum in pregnancy thus appears to be to support the implantation of the conceptus and its growth for a period thereafter, at the end of which it becomes dispensible. However, it continues to exist and function throughout the pregnancy, retaining all the necessary enzymes for steroid synthesis (Le Maire et al., 1968) and is capable of secretion as judged by differences in the progesterone concentrations in ovarian vein and peripheral plasma at different stages of gestation up to the 40th week (Mikhail and Allen 1967).

Plasma Progesterone Concentrations in Human Pregnancy:

The progesterone concentrations rise from implantation to the 5th week of gestation and then show a decreasing trend from 5th-9th week, the concentrations decreasing from 25 ng/ml to 17 ng/ml in the 9th week (Johansson 1969). From the 9th week of gestation progesterone concentrations then rise up to the 37th week from 20 ng/ml to 150 ng/ml (Tulchinsky et al., 1972) and reflect mainly placental synthesis of progesterone. At about the 37th week the concentrations plateau for the remainder of the gestation period.

Although progesterone concentrations in plasma continue to rise in pregnancy its concentration in the myometrium is reported to be fairly constant suggesting that the binding capacity of the myometrium is limited (Runnebaum and Zander 1971). This also suggests that the target tissues may not require all the progesterone that is synthesized during gestation.

Placental synthesis:

The human placenta synthesizes more progesterone than that of any other known species, 250 mg of the hormone being secreted per day (Van der Molen and AakVaag 1967). It possesses efficient side-chain cleavage, 3β -hydroxy steroid dehydrogenase and isomerase enzyme systems (Mason and Boyd 1972). Although the placenta can synthesize progesterone de novo from acetate (Van Leusden and Villee 1965), from pregnenolone and from its sulphate conjugate (Pion et al., 1966), these routes are quantitatively less important than the synthesis of progesterone from maternal cholesterol (Hellig et al. 1969). Placental progesterone does not depend on a contribution from the foetus because the progesterone concentrations in the umbilical vein far exceed

those from the umbilical artery and also that of the uterine artery (Harbert et al., 1964). Plasma progesterone levels (Lurie et al., 1966, Wiest 1967) or urinary pregnanediol levels (Cassmer 1959) in pregnancy, therefore, did not show significant alterations in the event of foetal death. Growth of the placenta is accompanied by a proportional increase in DNA (i.e. there is an increase in cell number of placenta). At about 35-36 weeks an increase in DNA ceases and this is accompanied by a slowing in placental growth rate. As the peripheral blood concentrations of progesterone plateau at about this period they may also closely reflect placental growth (Davies and Ryan 1972).

Role for Progesterone in Pregnancy:

Although progesterone, in association with oestrogens, promotes the growth of the uterus, its principle function in pregnancy is generally suggested to be to exert a "blocking effect" to reduce the excitation of uterine muscles resulting in inhibition of their rhythmic contraction-excitation activity (Csapo 1961, Davies and Ryan 1972, Heap et al., 1973). If the secretion of progesterone is drastically reduced or abolished by ovariectomy or by placental dislocation in pregnant rabbits (day 25 post

coitus) the contractile activity of the myometrium increases within 48 hours. If progesterone is injected the activity is abolished for 48 hours (Kumar 1967). Myometrial contractions of the human uterus can be inhibited by progesterone treatment in non-pregnant but not in the late pregnant or parturient subjects (Csapo 1969).

If the myometrial activity is inhibited in pregnancy by progesterone then at parturition, this activity should be associated with the removal of the inhibition by progesterone presumably because enough hormone is not available due to insufficient concentrations. However, high progesterone levels were measured in the uterine vein and peripheral plasma at a time of induced abortion after the intra amniotic administration of hypertonic saline (Short et al 1965) and in the peripheral plasma at labour (Yannone et al., 1969). It has nevertheless been shown that the myometrial cells in the vicinity of the placenta are less contractile than in the interplacental areas, and it is suggested that the growing volume of the uterus and its distance from the placenta i.e. "the volume to the progesterone ratio" may be a crucial factor in determining the progesterone block and the normal gestation period (Csapo 1969). Other factors such as plasma

protein-binding, metabolism, cytoplasmic "receptor" proteins may also be involved (Heap et al 1973) suggesting that regulation of myometrial activity at the cellular level may be a more complex phenomenon.

I N T R O D U C T I O N

CHAPTER 2

P R O G E S T E R O N E M E T H O D S

CHAPTER 2

PROGESTERONE METHODS

Initial attempts to carry out progesterone measurements were based on biological activity and were made by Clauberg et al. in 1933. They failed to detect progesterone activity in extracts from samples of between 235 and 335 ml of blood taken during various phases of the menstrual cycle (or during pregnancy). Attempts to detect progestational activity similarly failed even when 500 ml of human pregnancy blood or 70 ml of rabbit pregnancy blood were employed; these efforts were finally rewarded only when 8-12 litres of human pregnancy blood were used when a progestational activity equivalent to 1 rabbit unit of activity was measured.

The first successful attempt to measure progesterone in peripheral blood was made in 1954 when the isolation and chemical identification of the hormone from the blood of pregnant women was reported (Zander 1954). This determination was based on ultraviolet absorption at 240 nm, resulting from the presence of the $\triangle^4$ 3 oxo structure in the progesterone molecule. A sensitivity of 500 ng in 1 ml

plasma was obtained. In later developments of this method the sensitivity of the spectrophotometric detection was increased by the formation of progesterone derivatives with 2-3 fold increased molar absorbances resulting in sensitivities down to 40 ng (Watson et al. 1967).

Solutions of progesterone or its derivatives can be made to fluoresce by reaction with a strong acid or base. Based on this property, fluorimetric methods for determining the progesterone concentration in plasma have been developed (Short and Levit 1962, Heap 1964). Although fluorimetric methods are potentially more sensitive than spectrophotometric methods they also give high non-specific blank values which lower the sensitivity of the method. The lower limit of detection for accurate measurement is of the order of 10 ng (de Jong and Van der Molen 1970).

Because of their low sensitivities the spectrophotometric and fluorimetric methods are not suitable for measuring progesterone levels in non-pregnant individuals, for example, they are not able to distinguish between the progesterone levels in the follicular and luteal-phases of the menstrual cycle (Van der Molen and Aakvaag 1967).

Marked improvements in sensitivities occurred with the development of double isotope derivative (DID) and gas liquid chromatography (GLC) methods and with their emergence, progesterone could be reliably measured in the plasma of non-pregnant subjects. In the GLC system the stationary phase is a liquid film held on an inert solid support, and the mobile phase is a gas flowing continuously over the surface of the stationary phase. The concentration of the sample being assayed depends on its vapour pressure at the operating temperature which in turn is related to the boiling point and hence its molecular weight. Thus in the GLC system a volatile derivative of progesterone has to be formed and suitably purified. The vapours of this derivative in the mobile gas phase are blown over the stationary phase and these vapours travel at a rate which is proportional to the partition coefficient of each component in the sample between the two phases.

The DID technique, in which use is made of two radioactive tracers differing in their energy spectra, incorporates the technique of isotope derivative and isotope dilution. For example, a known amount of [^{14}C]-labelled progesterone is added to the plasma to correct for the

recovery losses. The plasma is extracted and the progesterone is purified. The purified extract is then reacted with a [^{3}H]-labelled reagent. The [^{3}H]-labelled progesterone derivative so formed is further purified. The amount of [^{3}H] radioactivity in the final residue is proportional to the amount of progesterone or its derivative. The accuracy and precision of the estimation is dependent on the ratio of the mass of [^{14}C] progesterone added (to correct for recovery) to the mass of progesterone present in the sample.

The detection limits for progesterone of the various GLC and DID methods as published vary from 1 - 10 ng (Van der Molen and Aakvaag 1967). Although these methods may be used for assessing luteal function, the amount of plasma they require and the number of extraction and purification steps involved to ensure the specificity of progesterone measurement make these two methods unsuitable for routine estimations of progesterone. However, they remain valuable as methods for the additional identification of progesterone.

More recently, the principles of competitive protein binding (CPB) (Murphy 1964) and radio immuno assay - RIA - Yalow and Berson 1960, Ekins 1960) have been adopted for the measurement of progesterone in the blood. With the application of these

techniques it has become practicable to estimate progesterone in multiple samples on a routine basis for physiological and clinical investigations. This is largely because of the vastly improved sensitivities of these techniques which have obviated the necessity of using the large amounts of plasma samples required in GLC and DID methods. Progesterone methods based on CPB and RIA techniques are also very much less laborious. The general principle of CPB and RIA techniques can be described as follows.

The hormone to be measured is allowed to compete with a trace quantity of radioactive hormone for the binding sites of a fixed mass of antibody or other specific protein. The saturation of the binding sites results in the partition of the hormone between bound and free fractions which are then physically separated. The change in the ratio of bound to free hormone, as shown by the distribution of radioactivity between the fractions reflects the total amount of hormone present. If the distribution of labelled hormone between the bound and free fraction of a set of standards is similarly determined then a standard curve can be constructed. The amount of hormone to be measured can then be quantitatively determined by comparison with the standard curve.

The binding protein most used in the CPB methods of progesterone is the plasma α_1- glycoprotein, transcortin or corticosteroid binding globulin (CBG). Pregnancy plasma which contains three times the normal concentration of CBG found in non-pregnant subjects has been used as a source of the binding protein after the removal of endogenous steroids. In addition, plasma from oestrogen treated normal or ovariectomised women; and plasma from normal or oestrogen treated dogs has been used as a source of CBG (de Jong and Van der Molen 1970).

The high sensitivities of progesterone-CBG assays are due to the high energy of the binding reaction of protein to progesterone. The association constants of human CBG-progesterone complex at $4^{\circ}C$ is 7×10^8 l/M (1 ml/450 pg) and decreases to 0.9×10^8 l/M (1 ml/3.5 ng) at $37^{\circ}C$ (Burton and Westphal 1972). CBG also binds certain other steroids with similar affinities and these include cortisol, corticosterone, 17 -hydroxyprogesterone, 11-deoxycorticosterone. Assays using CBG for the measurement of plasma progesterone therefore require a prior purification step (de Jong and Van der Molen 1970). Some such assays use a selective

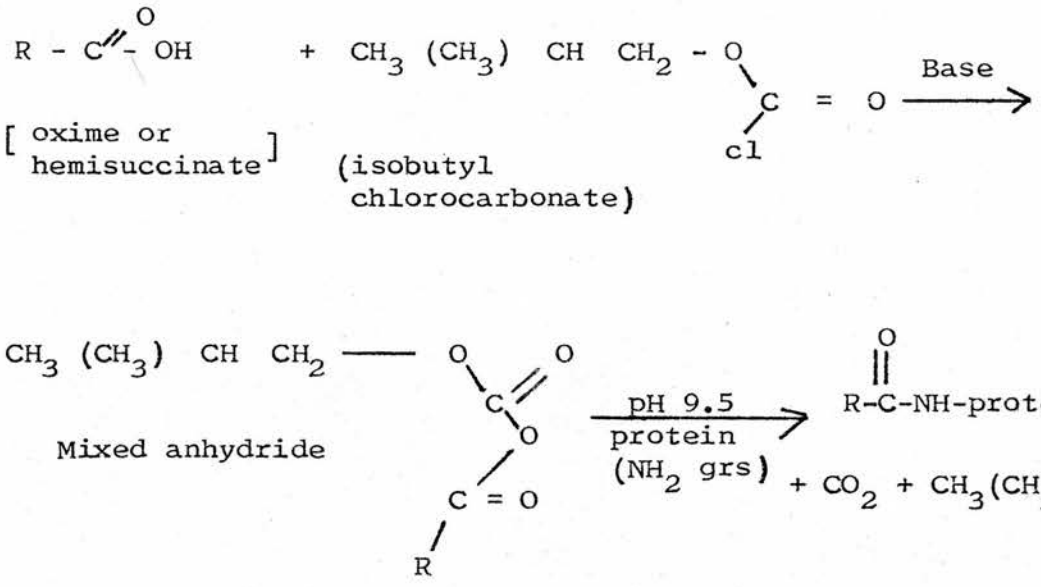
solvent extraction of progesterone into a non-polar solvent and the need for chromatographic purification is then obviated (Johansson 1969, Lurie and Patterson, 1970; Demetrious and Austin, 1971). The specificity of the progesterone measurement so achieved is adequate for monitoring luteal function but not for measuring follicular levels of progesterone as appreciable quantities of 17-hydroxyprogesterone (in particular) are extracted in a non-polar solvent

The other high affinity protein used in CPB methods for progesterone is the progesterone binding protein (PBP) which is synthesized during pregnancy in guinea pigs. It is a glycoprotein of α_2- or β globulin mobility, it has a mol. wt. 100000 and its plasma concentration 5×10^{-7}M. The association constant of PBP for progesterone at $4^{\circ}C$ is 4.8×10^{9} 1/M (1 ml/65 pg) and 3.9×10^{8} 1/M (1 ml/800 pg) at $37^{\circ}C$ (Burton and Westphal 1972). It discriminates well against cortisol, corticosterone and 17- hydroxyprogesterone but shows limited specificity towards 20 α-hydroxypregn-4-en-3-one, 5α -pregnane-3,20-dione, testosterone, 5α -dihydrotestosterone and deoxycorticosterone. Therefore for measuring progesterone levels such as those found in men and in the follicular phase a prior chromatographic purification is required. By combining the relative

specificity of PBP with the non-polar solvent extraction of progesterone, assay methods have been developed that are adequate for assessing luteal function without a chromatographic purification (Swain 1972, Pichon and Milgrom 1973).

The application of the RIA technique to progesterone measurement has resulted in improved sensitivities and specificities due to higher association constants and a higher specificity of antibody for progesterone. Compared to CPB methods, development of the RIA method is expensive, as it takes from weeks to months to raise an antibody of the required titre, sensitivity and specificity. RIA methods, however, prove much cheaper over long periods as many thousands of assays of progesterone can be performed over months or years with a single sample of antiserum. Because the binding protein has a lower association constant for progesterone than does the antibody it is relatively easier to separate the free and bound fraction using antibodies although equilibration times are slightly longer (minutes to hours) than those required in CPB assays (seconds to minutes). Thus RIA methods are more robust and versatile than CPB assays.

The studies of Landsteiner (1945) demonstrated that antibodies could be elicited to small molecular weight compounds by attaching them to large immunogenic protein carriers. The techniques for preparing steroid protein conjugates were later studied in detail (Goodfriend and Sehon 1958; Lieberman et al., 1959). Carboxylic acids were first attached to steroids by forming derivatives through the existing =O group (oximes) or -OH group (hemi succinates, chlorocarbonates) of the steroid nucleus (Lieberman et al., 1959). Steroids were then coupled to the protein through covalent bonds between the -COOH group of the steroid derivative and the ε-amino groups of lysene residues of the protein to form the biologically stable - CO-NH linkage. To prevent the cross linking amongst the -COOH group and $-NH_2$ group of the protein molecule during the coupling reaction, the steroid derivatives (containing the carboxylic group) were first activated to form the reactive intermediates (mixed anhydride, carbodi imide) e.g. as follows:



During the early period of development, steroid-protein conjugates were made by using the existing functional groups of the steroid molecule for coupling to the protein. The antisera so produced exhibited limited specificity, for example, antisera to testosterone raised by coupling at the 17-hydroxy position to BSA were only poorly able to detect structural changes embracing this part of the molecule whereas changes in the A ring were clearly recognized by these antisera (Beiser et al., 1959). These authors concluded that the portion of the hapten molecule farthest removed from the point of attachment to BSA determined to a great extent the specificity of an antiserum. In later studies (Midgley and Niswender 1970, Lindner et al.,

1970) conjugation of steroids to protein was performed at different positions of the steroid molecule and the specificities of antisera so produced was found to depend on the site of conjugation to protein. It was shown that conjugation to the protein at sites distal to the functional groups of the steroid molecule resulted in the production of antisera of higher specificity (Niswender and Midgley 1970) as all of the biologically active groups were then represented in the immunogen.

For the progesterone method to be presented in this thesis the selection of 11α-hydroxyprogesterone and 6 β-hydroxyprogesterone as the site for coupling to bovine serum albumin in the immunogens has been made on the basis of similar considerations. Hemisuccinates rather than chlorocarbonate derivatives of progesterone have been prepared. The longer link which they provided between the steroid and protein was considered to have afforded the steroid moiety a better chance of being clearly recognized in the conjugate. Conjugation of the 6 β and 11α-hydroxyprogesterone derivatives to bovine serum albumin was performed by the mixed anhydride reaction (Erlanger et al. 1957).

Since the work for this thesis was started in the beginning of 1971, 10 radio-immuno assay methods for measuring progesterone in human blood have been reported. In the first two published methods antisera were raised by coupling to the 21-position (Abraham et al., 1971) and to the 3-position (Furuyama and Nugent 1971) of the progesterone molecule. These antisera showed limited specificity for the steroids having structural differences from progesterone which were near the site of conjugation e.g. 17 hydroxyprogesterone, cortisol; and 5α-pregnane-3,20-dione and pregnenolone respectively. Consequently, in these methods a prior chromatographic purification of the progesterone was essential.

In other methods, antisera have been raised to the 11α-hydroxyprogesterone bovine serum albumin conjugate. As noted by Cameron and Scarisbrick (1973) the specificity of the antisera is not solely a function of the site of conjugation to the protein and these antisera which are raised against the 11α-position vary in their respective specificities to the same steroids. For example, the specificity of one antiserum to 11 - hydroxyprogesterone-BSA conjugate shows significantly

high cross reactions with 5α-pregnane-3,20-dione and 17-hydroxyprogesterone (Youssefnejadian et al., 1972) as compared to other reported antisera. A chromatographic purification of progesterone to be assayed is therefore necessitated when using the former antiserum. However, antisera to 11α-hydroxyprogesterone-BSA conjugates (as reported in all other methods) have generally exhibited a specificity which has allowed accurate progesterone measurements to be made without a chromatographic purification for plasma from men, postmenopausal women and the follicular-phase of the menstrual cycle.

Specific antibodies to progesterone have also been produced by coupling at the 6α-position (Niswender 1973, Jones and Mason 1974), 6β-position (Lindner et al., 1972, Riley et al., 1972, Niswender 1973, Jones and Mason 1974) and 7α-position (Bauminger et al., 1973), of the progesterone molecule. These antisera generally show appreciable cross-reactions with steroids such as pregnenolone and 5α-pregnane-3, 20-dione although in other respects their specificity is generally similar to that of antisera to 11α-hydroxyprogesterone-BSA conjugates.

Nevertheless, assay methods based on antisera raised to progesterone conjugated at the 6 or 7 positions have not been reported presumably because they suffer from greater non-specificity in this regard and do not provide any compensating advantage over those using antisera to the 11α-position.

SECTION 2

Methods

and

Materials

Materials

Radioactive steroids:

The following radioactive steroids were obtained from the Radio-Chemical Centre, Amersham, Bucks., U.K.:

	specific radioactivity
1α-2α- [(n) - ^{3}H] Progesterone	53 Ci/m mol
1,2,6,7- [(n) - ^{3}H] Progesterone	110 Ci/m mol
7α- [(n) ^{3}H] -17α-Hydroxyprogesterone	10.2 Ci/m mol
1,2- [(n) ^{3}H] - Cortisol	40 Ci/m mol
7α- [(n) ^{3}H] - Δ^5 - Pregnenolone	18 Ci/m mol

Following radioactive steroids were acquired from colleagues:

1α-2α- [(n) ^{3}H] 20α-Hydroxypregn-4-en-3-one	40 - 60 Ci/m mol
7 - [(n) ^{3}H] Androst-4-ene-3,17 - dione	6 Ci/m mol
7α- [(n) ^{3}H] - Dehydroepiandrosterone	10 Ci/m mol

Non-radioactive steroids:

Progesterone, 11∝-hydroxyprogesterone, 17-hydroxyprogesterone, corticosterone and cortisone were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and androst-4-ene-3,17-dione was from Organon Laboratories Ltd., Newhouse, Scotland, U.K: Progesterone, 11β-hydroxyprogesterone, 11-oxo progesterone, 11-deoxycorticosterone, 11-deoxycortisol (compound S), cortisol, dehydroepiandrosterone (DHA) and cholesterol were from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, U.K.: 6β hydroxyprogesterone, 5∝-pregnane 3,20-dione, 5β-pregnane 3,20-dione were from Steraloids, 140 Chichester Road, Croydon, U.K.: 20∝-hydroxypregn-4-en-3-one, 20 β-hydroxypregn-4-en-3-one, 3∝-hydroxy-5β-pregnan-20-one, 5∝-pregnane 3∝, 20∝-diol, 5∝-pregnane-3β,20∝-diol, 5β-pregnane-3∝, 20∝-diol pregnenolone (3 β-hydroxypregn-5-en-20-one) and progesterone were from the M.R.C. reference collection, Department of Chemistry, Westfield College, Hampstead, London N.W.3.

Biological reagents:

Freund's complete and incomplete adjuvant was purchased from Difco Laboratories, East Molesey, Surrey. The non-immune rabbit serum and the donkey anti-rabbit serum was from Wellcome Research Laboratories, Beckenham, England. Bovine serum albumin (BSA) Cohn fraction IV was from Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.

Reagents:

Sodium salts, Na_2HPO_4 and NaH_2PO_4 (BDH Anala R) were used to prepare the phosphate buffer. Silica gel (60 - 120 mesh), gelatin and sodium azide were BDH laboratory grade and all the other common laboratory chemicals were AR grade. Succinic anhydride, laboratory grade, was from Koch-light laboratories. Tri-n-butyl amine and isobutyl chloroformate (stabilised with calcium carbonate) was from Eastman, Kodak Ltd., Kirkby, Liverpool, U.K.

The other materials were as follows:

Sephadex LH 20 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Precoated (Silicagel F 254) thin layer chromatography plates were from Merck and chromatography

paper was Whatman paper no. 1. Cellulose (Microcrystalline for thin layer chromatography) was from E. Merck, AG, Darmstadt, Germany. Molecular sieve (aluminium sodium silicate) type 4A was BDH Laboratory grade.

Solvents:

Light petroleum (b.p. 40 - 60°C), pyridine, hexane, pentane were all BDH AnalaR grade and heptane was BDH laboratory grade. Light petroleum (b.p. 40 - 60°C), AnalaR grade; propylene glycol (propane 1,2,diol) and iso octane (2,2,4 - trimethyl pentane) both general purpose reagent grade were from Hopkins and Williams Ltd., Chadwell Heath, Essex, U.K. Ethanol (Absolute Alcohol B.P.) was from James Burrough Limited, 60 Montford Place, London S.E. 11. All the other organic solvents, AnalaR grade, were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Apparatus:

Hamilton syringes and barrels were from Hamilton, The Hague, Holland. Eppendorf micro pipettes were purchased

together with polypropylene tips from V.A. Howe Ltd., London. Cornwall automatic syringes were obtained from Becton Dickinson and Co., Rutherford N.J., U.S.A.,

The Heto Rotamix was manufactured by Birkerod Denmark. The reciprocating shaker (Barra) and the Multivortex mixer were from Baird and Tatlock, Freshwater Road, Chadwell Heath, Essex, U.K.

Glassware:

The radioactively contaminated glassware was soaked in a 5% solution of Decon (Decon Laboratories Ltd., Ellen Street, Portslade, Brighton, U.K.) for 12 - 24 h[illegible], then rinsed thoroughly in tap water and deionised water and after soaking for a further 12 - 24 h[illegible] in deionised double glass distilled water was dried in a hot air oven at 100°C. Other glassware was soaked for 12 - 24 h[illegible] in a solution of Pyroneg (Diversey Ltd., Cockfosters, Barnet, Herts.), after which it was rinsed thoroughly in tap water, deionised water and finally in deionised double glass distilled water before drying.

SECTION 2

METHODS

Preparation of Hemisuccinate and Conjugation to BSA

Preparation of the hemisuccinate derivatives:

Freshly distilled and KOH-dried pyridine (15 ml.) was added to a mixture of 1g of 11α-hydroxyprogesterone (3 m moles) and 10g of succinic anhydride (30 m moles) in a round bottomed flask containing washed antibumping granules. The flask was connected to a reflux condenser fitted with a calcium chloride trap to prevent moisture from entering the reaction flask. The contents of the flask were refluxed on a sand bath for a period of 15 - 16 hours. The reaction mixture was then evaporated under nitrogen under anhydrous conditions.

The dried semisolid residue was dissolved in 15 ml. of ethylacetate and the succinic acid removed by washing the organic layer 4 times with 8 ml. of water. The organic extract was evaporated and a 20 mg portion of the residue was checked by thin layer chromatography (TLC) on silica gel in the solvent system ethylacetate: methanol: acetic acid (90 : 7.5 : 2.5). This residue gave

2 spots, one of Rf 0.4 and the other of Rf 0.6, corresponding to the 11α-hydroxyprogesterone marker.

The crude hemisuccinate preparation was purified by dissolving in 15 ml. of ethyl acetate and extracting with an equal volume of saturated $NaHCO_3$ solution. The bicarbonate extract was rapidly adjusted to pH4 with dilute HCl to prevent hydrolysis of the 11α-hydroxyprogesterone hemisuccinate. The hemisuccinate was then extracted back into ethyl acetate (15 ml) and evaporated to dryness under nitrogen. A 20 mg portion of the residue when checked in the TLC system (as above) gave a single spot at Rf 0.4. The rest of this residue was dissolved in a minimum volume of hot methanol and the hemisuccinate crystallised by the addition of distilled water. After recrystallisation from methanol by addition of water the crystals were dried under vacuum and weighed. A yield of 35% was obtained.

A portion of the crystallised 11α-hydroxyprogesterone hemisuccinate was hydrolysed in I M NaOH, extracted into ethyl acetate and washed with water. The dry residue in the above TLC system gave a single spot (Rf 0.6) corresponding

to 11α-hydroxyprogesterone.

Preparation of 6 β-hydroxyprogesterone hemisuccinate:

1g of 6 β-hydroxyprogesterone (3m moles) and 10g of succinic anhydride (30m moles) were refluxed in 15 ml. of freshly distilled and dried pyridine for 8 h . The procedure for purification and crystallisation was the same as above. The Rf of the starting material in the above TLC system was 0.6 and that of the product 0.4.

Conjugation of 11α-hydroxyprogesterone and 6 β-hydroxyprogesterone hemisuccinates to bovine serum albumin (BSA):

The mixed anhydride reaction (Erlanger et al., 1959) was used.

Procedure:

11α-hydroxyprogesterone-bovine serum albumin conjugate:

To a mixture of 50 mg (0.15 m mole) of 11α-hydroxyprogesterone hemisuccinate and 70µl of tri-n-butylamine (55.7 mg, 0.3 m mole) was added 2.5 ml. of cold (4°c) dioxane, the reaction mixture being kept at 0°c.

15 µl of isobutyl chloroformate (17 mg, 0.15 m mole) was then added. If a precipitate persisted a further 5 µl of isobutyl chloroformate was added. The reaction was allowed to proceed for 30 min. BSA (170 mg, 2.5 µ mole), dissolved in a mixture of 4.5 ml. water, 3.5 ml. dioxane and 0.15 ml. NaOH, was then added while stirring, and the reaction mixture was kept at 0°C and the pH maintained at 8 by the addition of IM-NaOH.

The reaction mixture was then dialysed against running water overnight to remove the free steroids after which it was adjusted to pH 4.6 with IM-HCl. The resulting precipitate was allowed to stand at 4°C overnight and then centrifuged (2200g) at 4°C for 25 min. The residue was suspended in 5 - 10 ml. water and the pH adjusted to 7.5 - 8 to dissolve the product. Any free steroid was removed by precipitation with 8 ml. of cold acetone and adjusting the pH to 4.5. The precipitate was collected by centrifugation, resuspended in water (5 - 10 ml) and dissolved by adjusting the pH to 7.5 - 8. The residual free steroid and other impurities were removed by further acetone precipitation which was carried out for a total of 4 times. Each of the acetone extracts was evaporated, the residues dissolved in ethanol and scanned on an Unicam SP-800B spectrophotometer.

Under the conditions of investigation no steroid could be detected in the last two acetone extracts.

The protein conjugate from the above procedure was suspended in 8 ml. of water and dissolved by adjusting the pH to about 8. Insoluble material was removed by centrifugation (2200g) at 4°C for 25 min , the supernatant dialysed against running water overnight and then lyophilized. The yield of the conjugate was 115 mg.

Preparation of 6 β-hydroxyprogesterone-bovine serum albumin conjugate:

55 mg 6 β-Hydroxyprogesterone hemisuccinate (0.16 m mole) was reacted with 187 mg (2.7 mole) BSA (molar ratio 1 : 60). The entire reaction procedure was as above. A yield of 200 mg of 6 β-hydroxyprogesterone-BSA was obtained.

Production of Antibodies

(a) Immunisation Procedure:

New Zealand white rabbits were selected for immunisation.

Out of a total of 12 rabbits, 6 were immunised with 11α-hydroxyprogesterone-bovine serum albumin conjugate and 6 with 6 β-hydroxyprogesterone-bovine serum albumin conjugate. The animals were kept in standard conditions of light and darkness.

The conjugate (8 mg) was dissolved in 4 ml. of 0.9% saline and added to 4 ml. of Freund's complete or incomplete adjuvant. The two layers were thoroughly mixed by a blade homogeniser (MSE) until a stable emulsion was obtained. 0.1 ml. of the emulsion containing 1 mg. of conjugate was injected into each rabbit subcutaneously at 4 sites, 2 over the scapuli and 2 in the thighs.

(b) Injection Schedule:

All the primary injections were administered in Freund's complete adjuvant.

Immunisation of rabbits with 11α-hydroxyprogesterone-BSA conjugate:

All the booster injections of the conjugate were

prepared in Freund's incomplete adjuvant. After the 1st booster the rabbits were divided into two groups for further immunization.

In the first group, rabbits R1, R2 and R3 were boosted consecutively every $4\frac{1}{2}$ to 5 weeks up to the 3rd booster and were bled through the marginal ear vein 7 - 10 days after each injection. The rabbits were rested after the 3rd booster for about 3 months before a 4th booster was given. Rabbit R2 in this group was rejected after the 4th booster because the antisera obtained after each booster were consistently of low titres.

In the second group, rabbits R4, R5 and R6 were boosted about every 10th week and were bled 7 - 10 days after each booster. After the 3rd booster rabbit R6 died and Rabbits R4 and R5 were rejected because of poor titres of their antisera.

The two remaining rabbits R1 and R3 were rested for about $3\frac{1}{2}$ months before being given the 5th booster. Rabbit R3 then died and the remaining rabbit R1 was further boosted for the 6th, 7th and 8th times at intervals of 6, 20 and

46 weeks respectively. The full immunization schedule of the rabbits is given in Table 1 .

Immunization with 6 β-hydroxyprogesterone-BSA Conjugate:

The primary and all the booster injections of the conjugate were administered in Freund's complete adjuvant.

The rabbits were divided into two groups for immunization. Rabbits R 388, R 389 and R 391 were boosted consecutively every 6 weeks - and bled 7 - 10 days later. The other three rabbits R 379, R 390 and R 392 were boosted every 12 weeks although they were bled every 6 weeks along with the rabbits of the former group. The immunization schedule over a 30 week period is shown in the Table 2 .

(c) Collection and Storage of Antisera:

The rabbits were bled by an incision of the marginal ear vein, 50 ml. of blood from each animal being collected at a time. The tubes containing the whole blood were left at room temperature for 2 - 4 h and the resulting clot was freed from the sides of the container with a glass

Table [1] Immunisation of Rabbits with 11 ∝ -Hydroxyprogesterone-BSA Conjugate

Whether bled	Injection Interval weeks	Injection Schedule for Rabbits Male → M Female → F R1 (♂), R2 (♂), R3 (♂), R4 (♀), R5 (♂), R6 (♂)		Injection Interval weeks	Whether bled
No	0	Primary Injection		0	No
Yes	4·5	1st Booster		4·5	Yes
		R1, R2, R3	R4, R5, R6		
Yes	10	2nd Booster		10	No
Yes	14·5	3rd booster		14·5	No
No	20		2nd Booster	20	Yes
Yes	30	4th Booster R2 rejected R1 R3	3rd Booster R4 R5 rejected R6 dead	30	Yes
Yes	46	5th Booster R3 dead R1	-	-	-
Yes	52	6th Booster	-	-	-
Yes	72	7th Booster	-	-	-
Yes	118	8th Booster	-	-	-

Table 2 Immunization of Rabbits with 6 β-Hydroxyprogesterone-BSA Conjugate

Injection Schedule for Rabbits: Male → M; Female → F; R 388(♂), R 389 (♀), R 391 (♂)	Whether bled	Injection Interval weeks	Whether bled	Injection Schedule for Rabbits. Male → M Female → F R 379 (♂) R 390(♀) R 392 (♂)
Primary Injection	No	0	No	Primary Injection
1st Booster	Yes	6	Yes	1st Booster
2nd Booster	Yes	12	Yes	
3rd Booster	Yes	18	Yes	2nd Booster
4th Booster	Yes	24	Yes	
5th Booster	Yes	30	Yes	3rd Booster

rod. After this the blood was allowed to clot overnight at 4°C. It was then centrifuged at 2200g for 10 minutes either at 4°C or room temperature. The serum in the supernatant was collected and decomplimented by heating for 30 min at 56°C.

Sodium azide, NaN_3 was added as a bacteriostat to each pool of antiserum to a final concentration of 0.1%. The serum was then divided into 2 ml. portions in glass containers and stored at -20°C.

Radioactivity

Radioactive progesterone:

[1α, 2α- ^3H] progesterone (specific radioactivity 53 Ci/m mole) and [1,2,6,7 - ^3H] progesterone (specific radioactivity 110 and 81 Ci/m mole) were stored in dark brown bottles in redistilled ethanol at 4°C at a concentration of 10μ Ci/ml. The tritiated tracers were checked for homogeneity by partition chromatography on a column of Sephadex LH 20. The solvent system used was

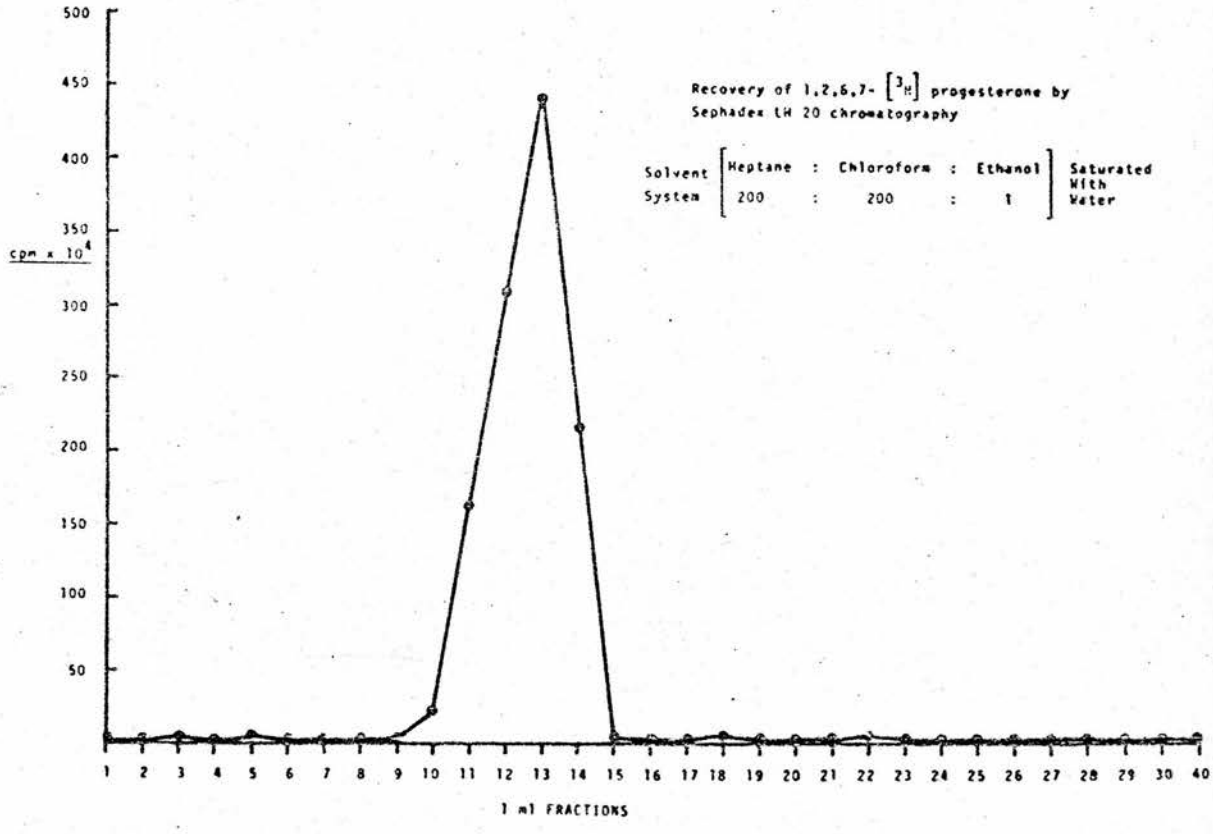
heptane-chloroform-ethanol (200 : 200 : 1 by vol.) saturated with water (Murphy 1971).

Procedure:

10g of the Sephadex LH 20 was swollen overnight in the organic phase of the solvent system and used to prepare a column of 31 cm x 0.9 cm (in glass columns fitted with a sinter (No. one porosity)). The column was washed with 200 ml of the organic phase before introducing the radioactive steroid. 1 ml fractions were collected. 85 - 95% of the progesterone was recovered in fractions 10 - 15 (fig. 1). The radioactive steroid so recovered was checked for homogeneity by TLC on silica gel in a methylene chloride - acetone (80 : 20 by vol) solvent system. The Rf was 0.6. It was also checked by paper chromatography in the Bush A system [light petroleum - methanol - water (100 : 96 : 4 by vol)]. The Rf was 0.89.

For routine assays the radioactive progesterone was used as supplied by the manufacturers although its homogeneity was checked from time to time by Sephadex LH 20

Fig. 1


Recovery of 1,2,6,7- [3H] progesterone by Sephadex LH 20 chromatography
Solvent System
Heptane : Chloroform : Ethanol
200 : 200 : 1
Saturated With Water
cpm x 10^4
500
450
400
350
300
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 40
1 ml FRACTIONS

chromatography. The chromatographically purified and unpurified [^{3}H] progesterone was further compared by use in standard curves.

Other radioactive steroids were used without further purification.

Radioactive counting:

Samples were counted in a Tracerlab columatic 200 or a Packard Tricarb liquid scintillation spectrometer. The tritiated progesterone to be counted was present in aqueous solution. This was mixed with 10 ml. of liquid scintillation fluid in counting vials which were then shaken for 2 min to extract the tracer into the organic phase. The radioactivity was counted without separation of the two phases (Abraham et al 1971). The samples were counted at 4°C or 25°C with a counting efficiency of 50 - 55%. 10,000 counts for the counting standards were collected which represented the amount of tracer added to each assay tube.

Solid-phase Antisera

Preparation of the covalently coupled antiserum:

1. Preparation of the activated microcrystalline cellulose:

Microcrystalline cellulose [5g] was suspended in distilled water to a final volume of 150 ml. Finely divided solid cyanogen bromide was then added [3g/5g of cellulose] while stirring. The pH of the suspension was maintained between 10.5 - 11 by the continuous addition of 2M NaOH until the reaction was complete as indicated by a constant pH at 11. Small pieces of crushed ice were added throughout the reaction to reduce the temperature. On completion of the reaction about 250g of ice were added and the mixture filtered on a sintered glass funnel. The activated cellulose was rapidly washed with 2·5 l of cold ($4^{o}C$) 0.1M $NaHCO_3$. This material was either coupled to antiserum at once or dried by washing with increasing concentration of acetone in water (500 ml of 50% then 70% acetone) and finally with acetone dried over a molecular sieve. The residual acetone was evaporated at room temperature and the dried active cellulose was sealed in glass ampoules under dry nitrogen and stored

at -20°C. Under such conditions it was found to be stable for many months.

2. Coupling of antibody to the activated microcrystalline cellulose

Antiserum (400µl) was diluted to 2.5 ml with 0.1M NaHCO3, 1g activated cellulose was added and mixture rotated end over end at 4°C for 3 days. After coupling, the solid preparation was washed essentially by the method of Wide (1969). The solid was washed 3 times with 50 ml of 0.1M NaHCO3 mixing for 20 min at room temperature for each wash. This was followed by a washing in 50 ml of 0.2 M acetate buffer pH4 for 60 min after which the solid was again suspended in the acetate buffer, subjected to ultrasonic dispersal (Soniprobe, Dawe Instruments Ltd., London) for 10 sec, rotated overnight or for 24 h and then centrifuged. This was followed by 3 washes in 0.1M NaHCO3 and a further two washes with 0.05M sodium phosphate buffer pH 7.5. Finally the mixture was washed twice with 50 ml of the assay diluent after which it was resuspended in the assay diluent and stored at 4°C for use. Under these conditions it was stable over a six month period.

Development of Radioimmunoassay method

Assay Medium:

Selection of water for the assay:

A suitable water for the assay diluent was selected from among the following:

(a) ordinary tap water

(b) deionised water

(c) sterilised water (autoclaved)

(d) double glass distilled water

(e) deionised double glass distilled water

The selection of the deionised double glass distilled water as the more suitable for assay diluent was made on the following basis: (1) A higher binding of [^{3}H] progesterone to the antiserum in the absence of unlabelled progesterone was obtained when the assay buffer used for the incubation mixtures was made from the double glass or deionised double glass distilled water as compared with that made from the other types of water. (2) 1 ml aliquots (x 10) each of double glass and deionised double glass distilled water samples were taken. These were extracted in the light petroleum (the solvent used in the method). The dried water extracts were then dissolved in

the respective diluent from the two types of water and their responses were measured from the standard curves prepared in the appropriate diluent. The apparent response given by the two types of water extracts (water blank) was minimal (less than 5g) or zero (same as the 0 std.) in the extracts of the deionised double glass distilled water as compared to double glass distilled water.

Buffers and the Assay Diluent

(1) Sodium salts Na_2HPO4 and NaH_2PO4 were used to prepare the phosphate buffer [of 0.0M and pH 7.5].

The assay diluent was 0.05M phosphate buffer pH 7.5 containing gelatine (0.1%) to minimise the adsorption of labelled and unlabelled progesterone to the glass surfaces. A fresh batch of diluent was prepared every week and stored at $4^{o}C$.

(2) TRIS (hydroxymethyl) aminomethane was used to prepare the TRIS-buffer 0.05M, pH 8.5.

Solubility of progesterone in the diluent:

An ethanolic solution of [^{3}H] progesterone containing 10, 100, and 600 pg progesterone was dispersed into glass tubes and taken to dryness under a stream of nitrogen or air. One ml of diluent was then added and the tubes mixed rapidly on a vortex mixer. Samples of the solution were taken after 2, 5, 10, 15, 30 and 60 min and the radioactive progesterone in solution measured. The results are shown in Table 3 . It was found that 95% or more of the [^{3}H] progesterone was recovered from the diluent in 2 minutes.

The preaddition to tubes (before the addition of ethanolic [^{3}H] progesterone solutions) of 0.5 ml of a 5mg/ml solution of propylene glycol in methanol (Clark and Gurpide 1972) did not improve the recoveries under the conditions of the experiment (Table 3).

Separation systems:

The bound fraction was separated from the free fraction by two methods.

Table 3 Solubility of [1,2,6,7 - ^{3}H] progesterone in diluent

Mass of [3H] progesterone	% Recovery (± S.D., n = 3) of Progesterone in Diluent over 60 min.					
	Time in Min.					
	2	5	10	15	30	60
10 pg without propylene glycol	96 ± 3	98.8 ± 3	99 ± 2	98.8 ± 2	98 ± 2	98.5 ± 1
10 pg with propylene glycol			98.6 ± 3			99.6 ± 2
100 pg without propylene glycol	95 ± 2	97.9 ± 3	97.8 ± 3	99 ± 3	97.8 ± 2	96 ± 2
100 pg with propylene glycol			99 ± 2			98 ± 3
600 pg without propylene glycol	96 ± 4	99 ± 2	98.5 ± 3	97 ± 2	96 ± 2	98.8 ± 4
600 pg with propylene glycol			97 ± 2			98 ± 1

(1) Double-antibody separation:

This was the previously optimised system (Hunter and Ganguli 1971) where the precipitation of the 1st antibody was achieved by the addition of a donkey antirabbit γ-globulin (DARS) in the presence of carrier non-immune rabbit serum (NRS). The dilutions of NRS and DARS for assays were selected from the flat plateau region of the precipitation curves. After the primary incubation of antigen and antibody for 2 h, 50 µl each of NRS and DARS was added and the tubes mixed after each addition. Complete precipitation was obtained after incubation with the second antibody for 16 h at 4°C. Diluent (1 ml) was then added before centrifugation to minimize non-specific adsorption of [^{3}H] progesterone, and the tubes were centrifuged at 1500 x g for 45 min at 4°C. The timing of centrifugation could be reduced to 15 min by the addition of talc (1 mg/ml) to the diluent. The supernatent containing the free fraction was counted. Using this system a separation of 95% or more could be achieved in the presence of excess of antibody and an apparent percent bound of 3 - 6% (non-specific binding) in the absence of the antibody.

(2) Separation of free and bound using covalently coupled antiserum:

After the 90 min incubation of the antigen and covalently-coupled antibody, 1 ml of diluent was added and the free and bound fractions were separated by centrifuging the tubes at 1500 x g for 10 - 25 min at 4°C.

The non-specific binding of [^{3}H] progesterone by the solid phase was negligible. When covalently-coupled non-immune rabbit serum was used instead of the antibody the non-specific binding of [^{3}H] progesterone to this was below 3%.

Dilution Curves and Standard Curves:

1. Dilution Curves:

Serial doubling dilutions of the antisera were made starting from an initial dilution of 1/500 of the antiserum. After the addition of 10 pg of [^{3}H] progesterone (50 µl) the tubes were incubated for 2 h (25°C) before the separation of the free and bound fraction by double

antibody. The same procedure was adopted for each solid-phase preparation starting from an initial dilution of 1/1000.

2. Standard curves:

Antisera dilutions that bound 70% or 50% of the [^{3}H] progesterone were selected from the dilution curves for use in the standard curves. Standard curves included 4 zero standards (binding of [^{3}H] progesterone to antibody in the absence of unlabelled progesterone) and duplicate progesterone standards containing 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560 pg amounts. Three non-specific blanks were also taken which, when separating by the double antibody method, did not contain the first antibody. For the solid phase assay system the non-specific blanks contained excess of progesterone (10 ng/ml) to saturate the antibody binding sites or, alternatively, the non specific blanks contained covalently-coupled non-immune rabbit serum instead of the first antibody.

(a) Double antibody system:

The tubes containing ethanolic progesterone standards were dried under nitrogen or air. Diluent was then added to all tubes and a time of 10 min was allowed for progesterone to dissolve. This was followed by the addition of [^{3}H] progesterone (10 pg in 50 µl) and then the antiserum (50 µl). The tubes were incubated for 2 h at 25°C and the free and bound separated by the addition of double antibody system.

(b) Solid phase:

The ratio of antiserum to cellulose was arranged so that each assay tube would contain sufficient antiserum to give 65 - 75% binding of the 10pg of tracer in an incubation volume of 1 ml when 50 - 100 µg of solid-phase antiserum was added. The tubes were incubated for 90 min on a reciprocating shaker (170 - 200 strokes/ min) and the bound and free fraction separated by centrifugation.

(3) Progesterone standards from different sources were compared using the antiserum to 11α-hydroxyprogesterone-BSA (R1 4th booster) selected for the routine assay (Table 4).

Table 4 Comparison of progesterone standards from different sources using 11∝-hydroxyprogesterone-BSA antiserum from Rabbit R1 (4th booster)

Standard curve		Unlabelled progesterone Source				Mean ± (S.D.)
		Koch-Light Labs % Bound	Koch-Light Chromatography % Bound	Sigma Chemical Company % Bound	MRC, Reference Collection % Bound	% Bound
5 pg	1	70.0	68.9	66.1	68.9	68.2 ± 0.52
	2	67.9	67.6	69.1	67.5	
10 pg	1	66.8	62.5	64.8	64.1	64.9 ± 1.5
	2	65.1	63.8	65.4	66.9	
20 pg	1	62.5	64.4	62.4	64.1	62.0 ± 1.55
	2	60.4	60.8	61.4	60.6	
40 pg	1	56.4	57.5	57.0	55.6	55.8 ± 1.29
	2	54.0	54.4	56.8	54.9	
80 pg	1	47.5	49.9	47.1	46.4	47.2 ± 1.3
	2	46.4	46.1	46.4	48.2	
160 pg	1	38.4	35.8	34.8	36.6	36.8 ± 1.7
	2	39.8	36.4	37.5	34.8	
320 pg	1	24.8	29.5	28.4	26.6	26.6 ± 0.7
	2	26.6	26.8	25.5	24.7	
640 pg	1	14.5	16.4	16.7	17.1	17.1 ± 1.6
	2	17.5	20.4	17.4	16.9	
1280 pg	1	8.4	11.4	6.8	11.6	9.1 ± 1.8
	2	7.0	9.8	9.4	8.6	
ostds	1	70.4				70.5 ± 0.52
	2	70.8				
	3	71.1				
	4	69.9				
non-specific blanks	1	5.8				5.6 ± 1.4
	2	6.8				
	3	4.1				

The purity of the progesterone standards from different sources was found to be similar as judged by the values for the different points on the standard curve.

(4) Table 5 shows the effect of temperature and time of incubation on the binding of 11α-hydroxyprogesterone-BSA antiserum (R1, 4th booster) to 1α, 2α [^{3}H] progesterone in the absence and presence of 20, 80, 320 pg amounts of unlabelled progesterone. From the values obtained (Table 5) it appears that equilibrium is attained in 30 min at 4^{o}C and 22^{o}C. However, as the separation of bound and free was achieved by the double antibody separation it is not known whether the primary reaction between antigen-antibody was stopped immediately on the addition of the second antibody reagents. The equilibrium was maintained over a 24 h period.

Procedure for the antisera specificity experiments:

Stock solutions of steroids were made at a concentration of 1 mg/ml in ethanol. A suitable portion was then diluted with the diluent to give a final concentration of 11μg/ml (containing 0.1% ethanol). All

Table 5 Effect of temperature and time of incubation on binding of [1α,2α-^{3}H] progesterone to Prog-11-AS- R1 (4th bleed)

% Bound

Assay tubes containing	0·5 h		1 h		2 h		4 h		8 h		16 h		24 h	
	Room temp	4°C	Room temp	4°C	Room temp	4°C	Room temp	4°C	Room temp	4°C	Room temp	4°C	Room temp	4°C
0 Stds n = 4	71±3	69±2	69±2.5	70±1.5	71.5±1.5	70±1.5	71±1	71.5±1	69.5±2	70±1.5	72±3	71±2	70±2	69.5±
20 pg n = 2	64.0	64.0	62.5	62.0	62.0	62.5	62.0	61.5	62.5	61.0	63.5	62.0	63.5	62.
80 pg n = 2	49.0	49.0	47.0	48.0	47.5	48.0	46.0	47.5	48.0	47.0	48.0	46.5	48.0	47.
320 pg n = 2	28.0	27.5	29.0	30.0	27.0	27.5	26.0	28.0	26.0	27.0	28.0	26.0	29.5	27.
Non-specific binding n = 2	5.0	3.0	4.5	3.5	4.0	5.0	5.0	5.0	6.5	4.0	5.5	6.0	6.5	5.

solutions were stored at 4°C and the solutions in diluent were discarded after one week.

Procedure:

A maximum of 8 compounds were tested with 3 different antisera in one experiment. For each steroid a range of 1 pg - 10 µg was covered by serial 10 fold dilutions of the starting concentration of 11 µg/ml. Free and bound were separated by double antibody system.

The percent cross-reaction was calculated as described by Abraham (1969). It is expressed as the mass of unlabelled steroid required to displace 50 per cent of the bound tracer.

Mass of [^{3}H] progesterone required to displace 50% = X

Mass of cross reacting steroid being displaced at 50% = Y

% cross-reaction = X/Y x 100

Extraction by solvents:

A series of organic solvents were investigated for extraction of progesterone from plasma as shown in the Table 6 . These were the non-polar solvents heptane (b.p. 98°C), hexane (b.p. 65°C) and pentane (b.p. 38°C) and light petroleum (b.p. 40 - 60°C). Diethyl ether (b.p. 35°C), a relatively polar solvent, has been used for extracting progesterone from plasma (Thorneycroft and Stone 1972) and isoctane (b.p. 99°C) has been used to elute progesterone from celite chromatography columns (Abraham et al 1971a). The selection of light petroleum (b.p. 40 - 60°C) as the solvent for extraction was based on its low boiling range and low cost. It was also easily available.

Purification of light petroleum:

A mixture of light petroleum and concentrated H_2SO4 (100 : 10 by vol) was stirred for 4 - 14 h . The acid was separated and washing of solvent with further batches of acid continued until the acid remained colourless after 6 h of stirring. The solvent was washed with $NaHCO_3$ (0.2M) to neutralise the acid then with deionised double

Table 6 Extraction of [^{3}H] progesterone from plasma by different organic solvents

Extraction in 200 µl of male plasma (n = 3) by the solvents	Volume of solvent used	% Extraction (mean ± S.D)	
		1st Extraction	2nd Extraction
Iso octane (2, 2,4-tri-methyl Pentane)	2 ml	84.6 ± 4.5	90.2 ± 1.85
Di ethyl ether	2 ml	81.2 ± 8.8	89.8 ± 4.3
Heptane	2 ml	87.8 ± 3.2	95.1 ± 0.6
Hexane	2 ml	86.4 ± 5.4	94.5 ± 1.3
n - Pentane	2 ml	88.3 ± 8.3	93.0 ± 4.85
Light Petroleum	2 ml	81.5 ± 9.4	88.5 ± 5.3

glass distilled water and was then dried over Na_2SO4 for 1 - 4 h. The dry solvent was passed through a 45 cm x 0.4 cm column of silica gel (800g) which had been heated at 100°C overnight. The column was fitted with a sinter of zero porosity. The purified solvent was washed twice with deionised double glass distilled water, dried over Na_2SO4 for 1 - 3 h and stored in dark brown bottles at room temperature.

Routine Extraction Procedure:

Plasma:

To a portion of plasma (or water) ethanol was added (10 vol. plasma + 1 vol. ethanol). After mixing on a Vortex mixer the tubes were left standing for 10 min. Extraction was carried out with 10 vol. or a minimum of 2 ml of light petroleum (b.p. 40 - 60°C) for 2 min by hand on a Vortex mixer or on a multivortex mixer which could mix simultaneously one hundred 15 mm x 75 mm glass tubes (for volumes up to 200µl plasma) or fifty 22 mm x 150 mm sized tubes (for volumes above 200µl plasma) without stoppering. After extraction the aqueous layer was frozen in a bath of solid CO_2: ethanol, the solvent extracts poured off and collected in the glass assay tubes (15 mm x 75 mm). The solvent was

evaporated under a stream of nitrogen or air in a heating block at 60°C, and heated at this temperature for a further 20 - 30 min.

Urine:

(0·5 ml)
liquots of urine collected during the follicular or luteal phases of the menstrual cycle were extracted with 10 vol. of light petroleum without the addition of ethanol. The rest of the procedure was the same as that for plasma.

Routine Assay Procedure:

A suspension of solid-coupled antiserum, 0·95 ml, (R1, 4th booster) was added to the dried extract residues at a concentration to bind about 70% of the labelled tracer. 10 pg of [^{3}H] progesterone in 50 µl of diluent was added to each tube and these were incubated on a reciprocating shaker at 170 strokes/min for 90 min at 20 - 30°C.

Assay Design and Quality Control:

[A.] All samples were assayed in duplicate. The plasma volumes were selected in relation to their expected progesterone responses on the standard curve. 500 µl, 200 µl, 10 - 100 µl portions were selected for measuring levels in men or postmenopausal women, follicular phase plasma and for luteal phase plasma respectively. The plasma volume was made up to 100 µl with deionised double glass distilled water.

Two standard curves were set up with each assay, one extracted and the other unextracted. To all tubes of the extracted standard curve containing dry ethanolic residues of progesterone standards a 200µl portion of deionised double-glass distilled water was added. After mixing, the tubes were left standing for 10 min before extraction with light petroleum by the standard procedure.

The following controls were also included in each assay:

(a) The recovery by the extraction procedure was checked in quadruplicate. 200µl aliquots of a male plasma pool were added to dry ethanolic residues of 10 pg of [^{3}H] progesterone. The tubes were mixed and left standing for 10 min before extraction. The recovery of radioactivity in the extract was measured.

(b) The assay end-point recovery was checked by adding unlabelled progesterone to plasma. 200µl portions of a male plasma pool were added to dry ethanolic residues of 80, 160 and 640 pg of unlabelled progesterone. The tubes were left standing for 10 min after mixing and

were then extracted and assayed.

(c) A 100µl portion of luteal phase plasma diluted with male plasma was used as a quality control plasma. One triplicate set of the quality control plasma was assayed at the beginning and one triplicate set at the end of each assay.

(d) Zero standard tubes (4-6) were scattered throughout the assay to monitor the binding of tracer to antibody in the absence of unlabelled progesterone.

Urine

0.5 ml portions was taken for assaying progesterone levels in follicular and luteal phase urines. 0.5 ml portions of a pool of luteal phase urine were used as quality controls. The rest of the assay design was similar to that of plasma.

[B.] Assay Specificity:

Plasma:

This was checked by comparing progesterone levels in chromatographically purified and unpurified extracts of plasma. 500µl portions of plasma from the follicular phase, 50µl portion of plasma from the luteal phase and 100µl of quality control plasma samples were each taken in quadruplicate. 500µl aliquots of follicular phase plasma were also added to 4 tubes containing the dry ethanolic residues of 500 pg of unlabelled progesterone and to another 4 tubes containing similar residues of 100 pg of [1α, 2α - ^{3}H] progesterone to monitor the recovery by the extraction procedure and the chromatography procedure. After extraction, duplicate samples from each category containing the dry residues were chromatographed by Sephadex LH 20 chromatography as described under the purification of radioactive progesterone (P. 67) and fractions 10 - 15 (5 ml) were collected. Portions of 5 ml eluate from a separate column were added to each tube of the extracted standard curve. The dry residues in all tubes were assayed as described.

Urine:

Quadruplicate 0.5 ml portions of the urine pools from the follicular and from luteal phase were taken. Another 0.5 ml portions of the follicular phase urine were also added to 4 tubes containing dry residues of unlabelled progesterone (500 pg) and also to other 4 tubes containing residues of 100 pg of [1α, 2α-^{3}H] progesterone. These were chromatographed as above and fractions 15 - 21 (6ml) were collected.

[C] Unextracted plasma:

The progesterone responses in extracted and unextracted plasma samples using covalently coupled 11α-hydroxyprogesterone-BSA and 6β-hydroxyprogesterone-BSA antisera were compared as follows. For extracted samples neat plasma was used whereas the unextracted samples were assayed as (a) neat plasma (b) plasma diluted x 4 times (c) plasma diluted x 4 times, heated for 1 h at 100°C, centrifuged (1500 x g) at 4°C for 10 min and supernatant assayed. Plasma pools from males and postmenopausal women were assayed at dose levels of 50µl, 100µl and 200µl volumes and those from luteal phase and quality control pools were assayed at dose levels of 20, 50, 100 and 200µl. The incubation vol of all tubes, including standard curves and extracted samples, was first adjusted to 200µl before the addition of the respective antisera. The assay procedure was as described.

SECTION 3

Results and Discussion

1. Steroid antigens and conjugation to the bovine serum albumin (BSA).

Formation of hemisuccinate derivatives of 11α-hydroxyprogesterone and 6 β-hydroxyprogesterone (figs.2,3) were confirmed by the following methods.

1. Infra red spectrum:

The spectra of the starting steroids and the products are shown in figs.4,5 . The peak given by the carbonyl group of the ester bond at 1733^{cm-1} is present in the products but not in the original steroids.

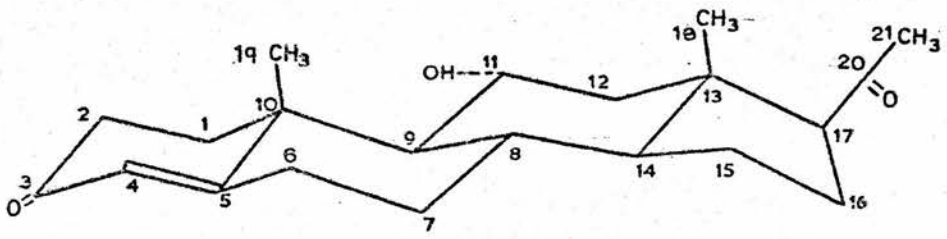
2. Nuclear Magnetic Resonance (NMR) spectra:

The maximum absorption of the starting materials and their products were interpreted as shown in table 7 . The evidence of hemisuccinate formation was obtained from spectral shifts and the appearance of peaks of methylene (CH_2) groups and changes in the signal which are consistent with the expected substitutions at the 11β - and 6α-positions.

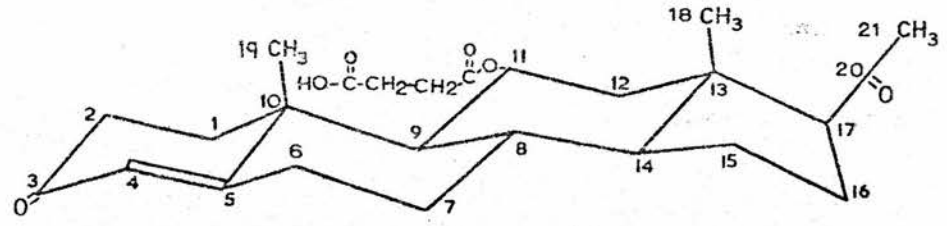
3. Melting points (M.P.) and Thin Layer Chromatography (TLC):

The M.P. of 11α-hydroxyprogesterone and 6 β-hydroxyprogesterone were respectively 160^{o}C and 165^{o}C

Fig. 2



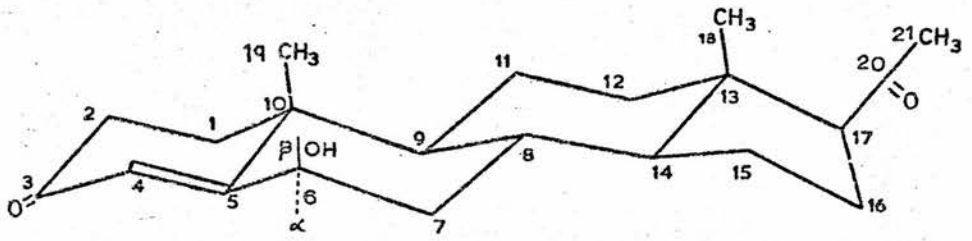
11α-HYDROXY PROGESTERONE M.P. 160°



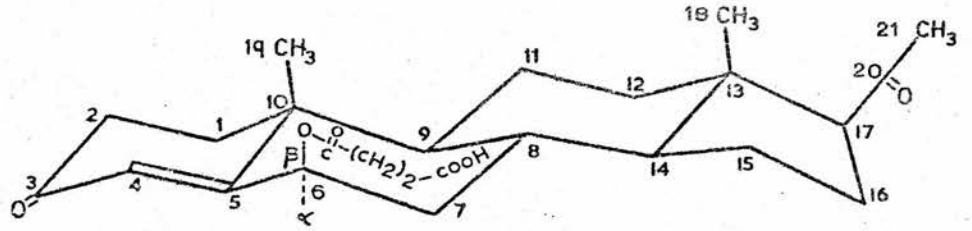
11α-HYDROXY PROGESTERONE HEMI SUCCINATE M.P. 148°

T.L.C. SYSTEM	:	A	Ethyl Acetate	:	MeOH	:	Acetic Acid	:	B	:	Benzene	:	EtOH
			90		7.5		2.5				4		1

Fig. 3



6β-HYDROXY PROGESTERONE M.P. 165°



6β-HYDROXY PROGESTERONE HEMI SUCCINATE M.P. 155°

T.L.C. SYSTEM :	Benzene	: Ethyl Alcohol	: Ethylacetate	: MEOH	: Acetic Acid
	4	I	90	7.5	2.5

Fig. 4

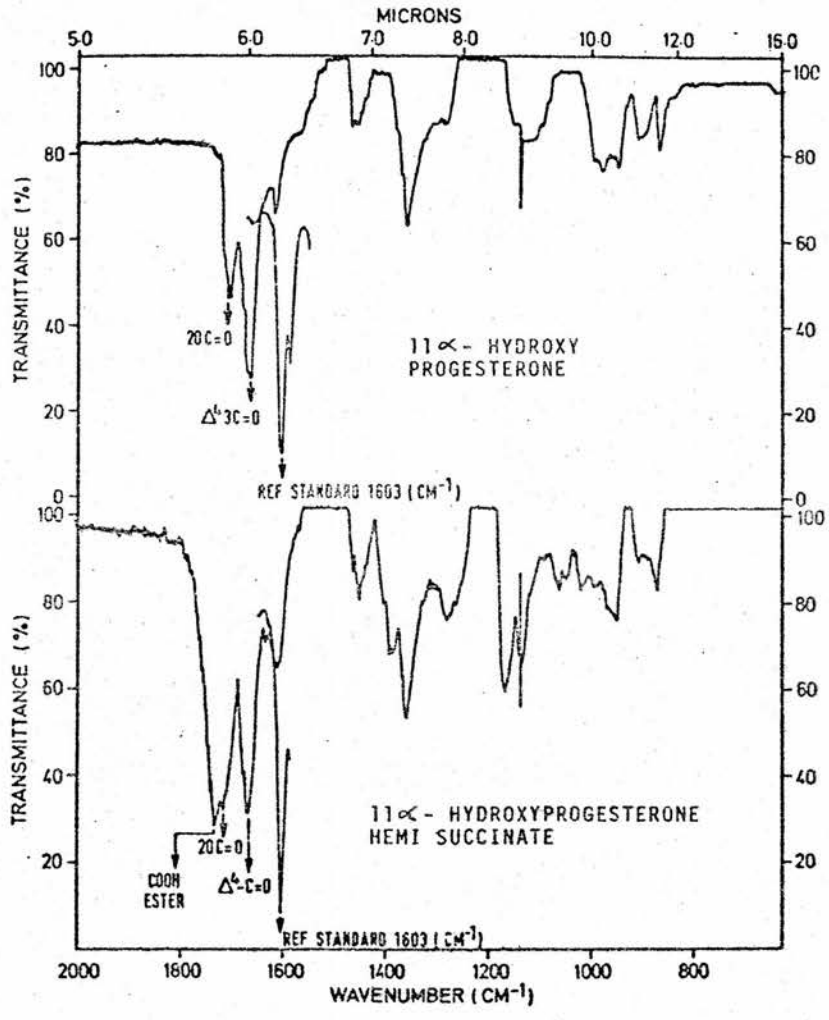

MICRONS
5·0
6·0
7·0
8·0
10·0
12·0
15·0
TRANSMITTANCE (%)
20C=O
Δ⁴3C=O
11∝- HYDROXY PROGESTERONE
REF STANDARD 1603 (CM⁻¹)
11∝- HYDROXYPROGESTERONE HEMI SUCCINATE
COOH ESTER
Δ⁴-C=O
2000
1800
1600
1400
1200
1000
800
WAVENUMBER (CM⁻¹)

Fig. 5

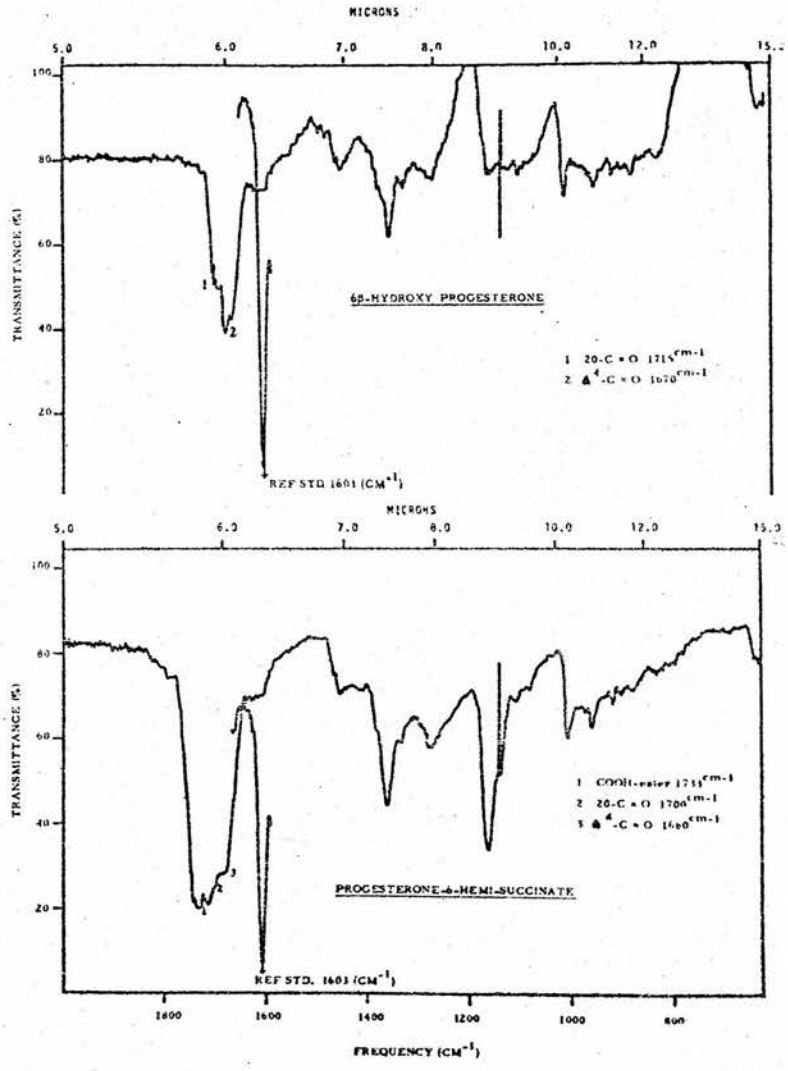

MICRONS
5.0
6.0
7.0
8.0
10.0
12.0
TRANSMITTANCE (%)
100
80
60
40
20
6β-HYDROXY PROGESTERONE
1 20-C = O 1715 cm-1
2 Δ4-C = O 1670 cm-1
REF STD 1681 (CM-1)
MICRONS
5.0
6.0
7.0
8.0
10.0
12.0
15.0
TRANSMITTANCE (%)
100
80
60
40
20
1 COOH-ester 1715 cm-1
2 20-C = O 1700 cm-1
3 Δ4-C = O 1660 cm-1
PROGESTERONE-6-HEMI-SUCCINATE
REF STD. 1663 (CM-1)
1800
1600
1400
1200
1000
800
FREQUENCY (CM-1)

TABLE 7

Nuclear Magnetic Resonance Spectrum Analysis of 11α -hydroxy progesterone, 6- β hydroxy progesterone and their hemisuccinates

	11α -hydroxy progesterone	11α -Hydroxyprogesterone hemisuccinate
C-18 methyl group	0.71 δ	0.69 δ
C-19 methyl group	1.25 δ	1.33 δ
C-21 methyl group	2.09 δ	2.13 δ
C-11- β -H	5.10 - 5.50 δ as complex multiplate	3.90 - 4.20 δ as sextet
C-4-H	5.76 δ	5.73 δ
CH_2 groups of hemisuccinate	Absent	2.48 δ

	6- β -hydroxy progesterone	6 β -Hydroxyprogesterone hemisuccinate
C-18 methyl group	0.70 δ	0.70 δ
C-19 methyl group	1.38 δ	1.29 δ
C-21 methyl group	2.12 δ	2.12 δ
C-6-α -H	4.36 δ as triplet	5.46 δ
C-4-H	5.81 δ	5.93 δ
CH_2 groups of hemisuccinates	Absent	2.64 δ

while those of their hemisuccinates were 148°C and 155°C. Mobilities on TLC in the solvent system ethylacetate: MeOH: acetic acid (180:15:5) were 0.4 for the hemisuccinates and 0.6 for the starting material. Hydrolysis of the crystals of the respective hemisuccinate preparations yielded compounds with Rf values (0.6) similar to those of the starting materials.

The general approach of making hemisuccinate derivatives was taken from Erlanger et al. (1959) who described a hemisuccinate preparation at the 21 position of deoxycorticosterone. Using their ratios of steroid: succinic anhydride (1:3) and refluxing time of 4.5 h only a 10% yield was obtained. The yield was similarly poor after refluxing for 24 h . This may have been due to the saturation of pyridine with moisture over such a long period resulting in its inability to continue to absorb the succinic acid formed during the reaction. This may also have been the reason for the poor yields (less than 10.0%) which resulted when the reactants were left at room temperature for 3 - 35 days.

The reaction mixture ratios were therefore altered to 1 : 10 (steroid-succinic anhydride) and the reaction

carried out by refluxing for 4.5, 8.0 and 16 h .
A reaction time of 16 h was found to give the highest yield (35% as compared to 20 - 25%). The use of freshly distilled pyridine was most crucial in obtaining high yields.

As the 6 β-position is nearer than the 11∝ - to the double bond at Δ^4 -3 oxo position (allylic) it is more reactive and therefore for the preparation of 6 β -hydroxyprogesterone hemisuccinate a shorter refluxing time period of 8 h was arbitarily chosen (40% yield).

Conjugation of 11∝ -hydroxyprogesterone hemisuccinate and 6 β-hydroxyprogesterone hemisuccinate to bovine serum albumin (BSA) was achieved by the mixed anhydride reaction (Erlanger et al., 1959). After the conjugation unreacted progesterone-derivative was separated and removed from the progesterone-BSA conjugate by repeated acetone washings and the removal of free progesterone checked in the washings by u.v. scanning as described under methods.

Formation of 11α-hydroxyprogesterone-BSA and 6 β-hydroxyprogesterone-BSA conjugates was confirmed by evidence obtained from ultra violet (u.v.) spectroscopy as shown in figures 6 and 7 . Judged on this evidence, the substitution of steroid molecules calculated on a molar basis were 18 steroid mols/mol BSA for the 11α-hydroxyprogesterone-BSA and 31 mols/mol BSA for the 6 β-hydroxyprogesterone-BSA conjugate. As progesterone absorbs strongly in the u.v. region because of the presence of the Δ^4 - 3 oxo structure it was not considered necessary to monitor the conjugation by an alternative method such as the addition of radioactive progesterone to the conjugation mixture.

It has been suggested that steroid-protein conjugates with molar ratios of less than 10 are not effective for antibody production whereas a steroid:protein molar ratio of 20:1 or above is most effective (Niswender and Midgley 1970). However no formal evidence correlating differences in substitution ratios with differences in the titre, avidity or specificity of antisera has been published.

Conjugation of 11α-hydroxyprogesterone and 6 β-hydroxyprogesterone derivatives to BSA has been achieved

Fig. 6

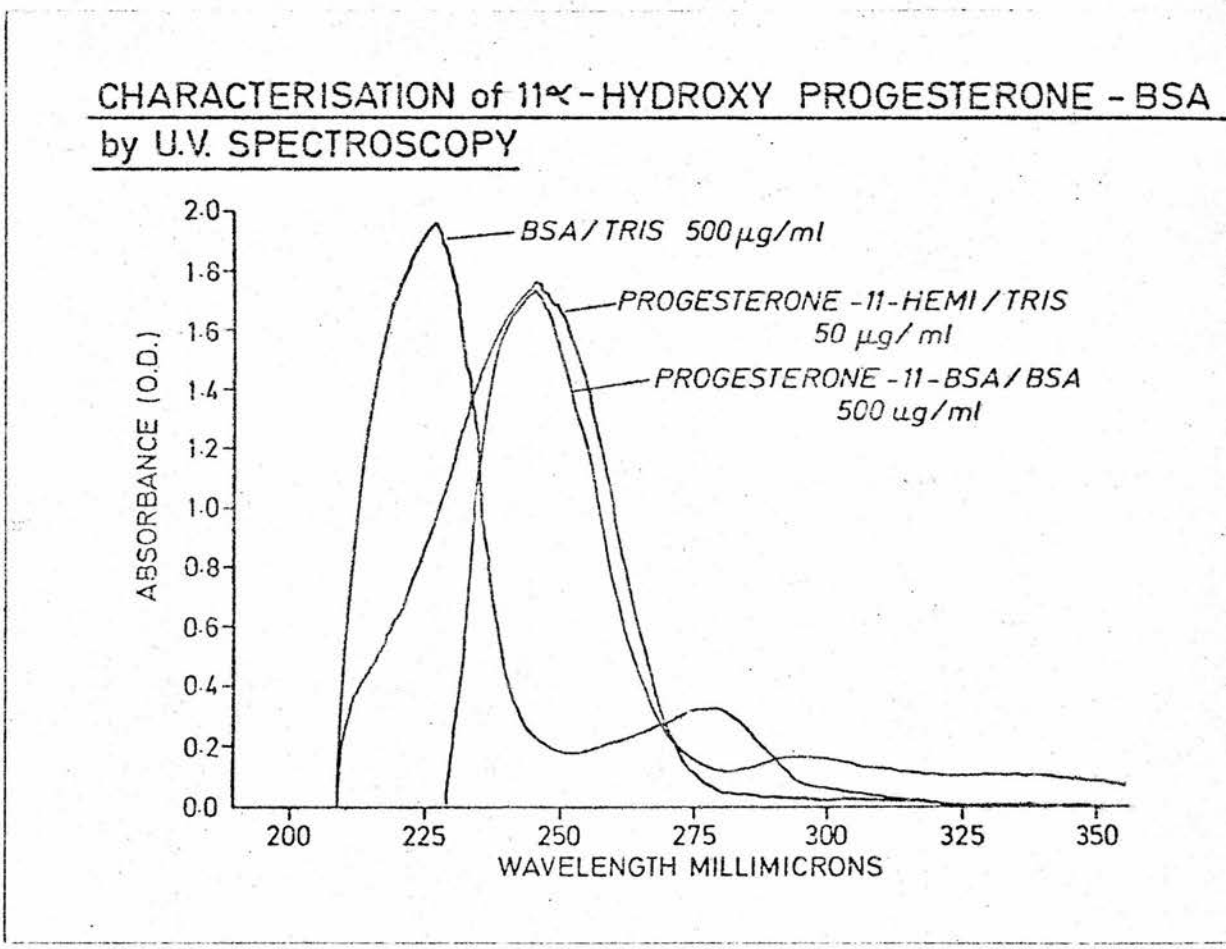
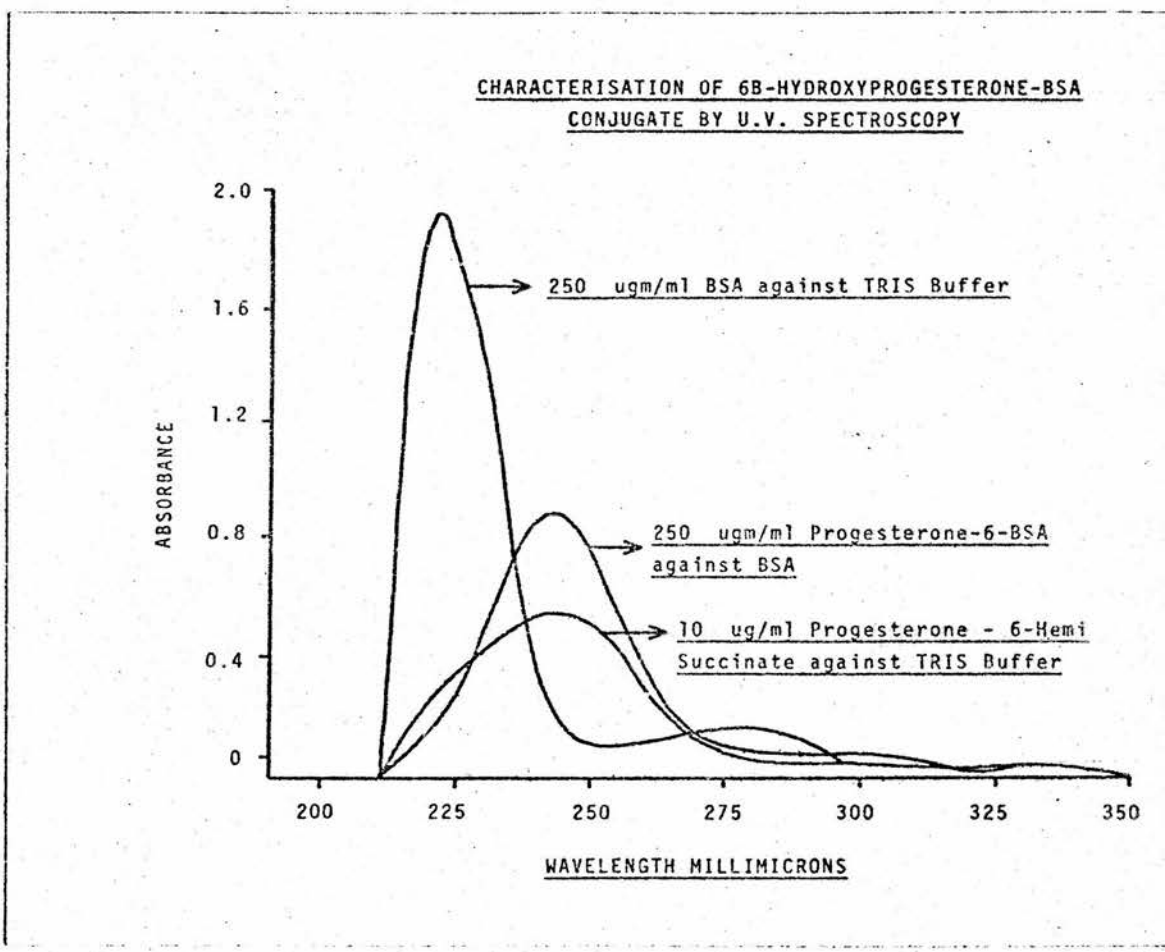

CHARACTERISATION of 11∝-HYDROXY PROGESTERONE - BSA
by U.V. SPECTROSCOPY
BSA / TRIS 500 µg/ml
PROGESTERONE -11-HEMI / TRIS
50 µg/ml
PROGESTERONE -11-BSA / BSA
500 ug/ml
ABSORBANCE (O.D.)
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
200
225
250
275
300
325
350
WAVELENGTH MILLIMICRONS

Fig. 7


CHARACTERISATION OF 6B-HYDROXYPROGESTERONE-BSA
CONJUGATE BY U.V. SPECTROSCOPY
ABSORBANCE
2.0
1.6
1.2
0.8
0.4
0
250 ugm/ml BSA against TRIS Buffer
250 ugm/ml Progesterone-6-BSA
against BSA
10 ug/ml Progesterone - 6-Hemi
Succinate against TRIS Buffer
200
225
250
275
300
325
350
WAVELENGTH MILLIMICRONS

by using different conjugation procedures and the steroid/protein substitution ratios obtained by these procedures is shown in Table 8 . Conjugation of progesterone derivatives to bovine serum albumin takes place through formation of peptide bonds to the Σ-amino group of the lysine residues. There are 59 lysine residues per BSA molecule and one N-terminal amino acid - that is a total of 60 free amino groups available for conjugation to the steroid derivative (Erlanger et al. 1957). From Table it appears that not more than two-thirds of the amino groups are available for conjugate to the steroid. This may be due to destruction of some of these groups during the conjugation - reaction procedure, to some type of chemical inhibition, or most likely to some of the $-NH_2-$ groups being rendered inaccessible because of the tertiary structure of the protein molecule (Erlanger et al. 1957).

TABLE 8

Conjugation of 11α -Hydroxyprogesterone derivatives to bovine serum albumin by different procedures

Derivatives of progesterone made at 11α -position	Method of Conjugation used	Substitution ratio progesterone mol/ mol BSA	Authors
11α -Hydroxyprogesterone hemisuccinate	Mixed Anhydride reaction	24 mols/mol BSA	Thorneycroft and Stone 1972
" "	" " "	18 mols/mol BSA	Spielar et al 1972
" "	" " "	26 mols/mol BSA	Youssefnejadian et al.1972
" "	" " "	20 mols/mol BSA	Kutas et al. 1972
" "	" " "	18 mols/mol BSA	Present method
" "	Carbodimide reaction	28 mols/mol BSA	Lindner et al. 1972
11α -Hydroxyprogesterone chloro-carbonate	Schotten-Baumann reaction	36 mols/mol BSA	Niswender 1973

Conjugation of 6 -hydroxyprogesterone-derivatives to bovine serum albumin (BSA)

Derivatives of progesterone made at 6 β -position	Method of Conjugation used	Substitution ratio progesterone mol/ mol BSA	Authors
6 β -Hydroxyprogesterone hemisuccinate	Mixed Anhydride reaction	28 mols/mol BSA	Niswender 1973
" "	" " "	31 mols/mol BSA	Present method
6 β Carboxymethyl progesterone	" " "	35 mols/mol BSA	Riley et al 1972
6 β Carboxymethyl progesterone	" " "	24 mols/mol BSA	Jones and Mason 1974
Progesterone 6 - (carboxymethylene) thioether	Carbodimide reaction	30 mols/mol BSA	Lindner et al 1972

SECTION 3

Results and Discussion

2. Immunization.

Primary injections of the conjugate were given in Freund's complete adjuvant at four subcutaneous sites - two in the inner aspects of the thighs and two over the scapuli. Using this route several groups of lymph nodes are sensitized and the responses are generally superior to those obtained by other simple procedures involving the intraperitoneal or intravenous routes (Hurn and Landon 1971). The dose of conjugate per rabbit has been kept at 1 mg for all injections.

Following the primary injections, booster injections were given at the same sites either in Freund's complete adjuvant (in the immunization of 6 β-hydroxyprogesterone antisera) or in Freund's incomplete adjuvant (in case of 11α -hydroxyprogesterone antisera). Although the route of immunization was based on the previous experience of the laboratory the advantage of boosting at the same sites may be that greater concentrations of sensitized cells are present as a result of primary injection at these sites (Parker 1971).

The schedule used for the first 30 weeks of immunization for the six rabbits immunized with

11α-hydroxyprogesterone-BSA conjugate is shown in fig. 11 (P. 121). After the primary injection in Freund's complete adjuvant (FCA), booster injections in Freund's incomplete adjuvant (FIA) were given to three rabbits (R1, R2 and R3) at intervals of 5 - 6 weeks up to the third booster and the other three rabbits (R4, R5 and R6) were boosted twice at intervals of 11 - 12 weeks. A further booster was given to rabbits of both groups after a rest of 12 - 15 weeks. Rabbits R2, R4 and R5 were rejected for giving unacceptable titres and Rabbit R6 died after 30 weeks. Rabbits R1 and R3 were further boosted at the 46th week after which Rabbit R3 died. Rabbit R1 was further boosted at 52, 72 and 118 weeks and the results are shown in fig. 12 (p.122). The immunization schedule for progesterone -6β immunised rabbits covering a period of 34 weeks is shown in fig. 13 (P. 123). Three of these rabbits (R388, R389 and R391) were boosted at 5 - 6 weekly intervals and the other three rabbits (R379, R390 and R392) were boosted at 12 - 15 weekly intervals, the primary and booster injections being given in Freund's complete adjuvant.

The choice of identical sites for primary and booster injections may not be important since other workers have obtained satisfactory antibodies by purposely changing the route of administration of the immunogen for the booster injection. For example, Thorneycroft and Stone (1972) gave primary injections of progesterone-BSA conjugate by the subcutaneous routes and booster injections either by the subcutaneous or intravenous routes. Kutas et al. (1972) gave primary injections in three front and three back toe pads, the routes were changed to intraperitoneal in the 2nd week, to intramuscular (4 different places) in the 3rd week followed by subcutaneous booster injections. The progesterone antisera produced by these above procedures by Thorneycroft and Stone (1972) and Kutas et al. (1972) respectively were of high sensitivity and good titre.

In contrast to the present immunization schedule, in almost all of the published radioimmuno assays of progesterone (Abraham et al. 1971a, Furuyama and Nugent 1971, Thorneycroft and Stone 1972, Kutas et al., 1972, Spieler et al. 1972, De Villa et al., 1972, Yousseffnejadian et al., 1972a) antisera have been raised by giving weekly

injections of conjugates in Freund's adjuvant for periods up to 3 or 6 weeks. Antisera so produced by these workers, except those by Thorneycroft and Stone (1972) and Kutas et al. (1972), are generally inferior in terms of titre and sensitivity when compared to the antisera raised by the present immunization schedule where the injections have generally been spaced at 5 - 6 or 11 - 12 weekly intervals.

Comparison of the present immunization schedule with those of others with respect to the quality and quantity of antibodies produced is difficult because there are many variations in these immunization procedures. The progesterone antisera raised by Thorneycroft and Stone (1972) and Kutas et al (1972) although raised by a different immunization procedure are similar to the present antisera in terms of their titre and sensitivity. The injection dose of 1 mg/rabbit used by these workers is, however, the same as used in the present procedure. The injection doses of progesterone-BSA conjugate used by Spieler et al. (1972) in their immunization procedure are higher than the injection doses of all the other workers. They injected 10 mg of conjugate/animal in FCA every week for 3 weeks before giving booster injections of 1 mg/animal at

monthly intervals. Although there were other variations in their immunization procedure, (for example, they gave primary injections in the foot pad and booster injections through the marginal ear vein), the poor detection limit (300 pg/ml) and lower titre (1/480) of their antisera as compared with all the others in the published studies was probably due to the high injection doses of immunogens that they used. It has been shown that large doses of immunogens have the capacity to induce tolerance in those cells which contain, and can potentially secrete high affinity antibodies. Such tolerance can prevent them from further proliferation (Goidl et al., (1968)).

In contrast, low doses of conjugate have been shown to be beneficial in eliciting antisera of high titre and affinity especially when injected intradermally at multiple sites. Thus 75-100 µg of testosterone-3-conjugate was adequate to elicit antibodies whose affinities were of the order of 1.4×10^9 l/m with titres ranging from 1/10000 - 1/60000 in 4 - 5 weeks without further boosting (Vaitukaitis et al., 1971). The authors could not distinguish whether their success was due to the use of multiple sites and the intradermal route or, to the minute concentrations of immunogen

injected. Lader et al. (1973) have compared the method of multiple intradermal injection with that of the standard sequential intramuscular injections in rabbits using 4 different immunogens (Thyroxine-BSA, Oestradiol-BSA, Angiotension I and h-TsH). Similar doses of immunogens were used for primary and booster injections, the rabbits were bled at intervals throughout the immunization schedule and antisera tested for titre and avidity. The results obtained with both methods were comparable except that slightly better titres were obtained with intradermal injections. Maximum titres were obtained within 10 weeks after intradermal injections and remained unchanged on boosting. Thus, as suggested by these authors and by Hurn (1974), the technique of multiple intradermal injections is preferable if rapid antibody production from limited amounts of immunogens is required.

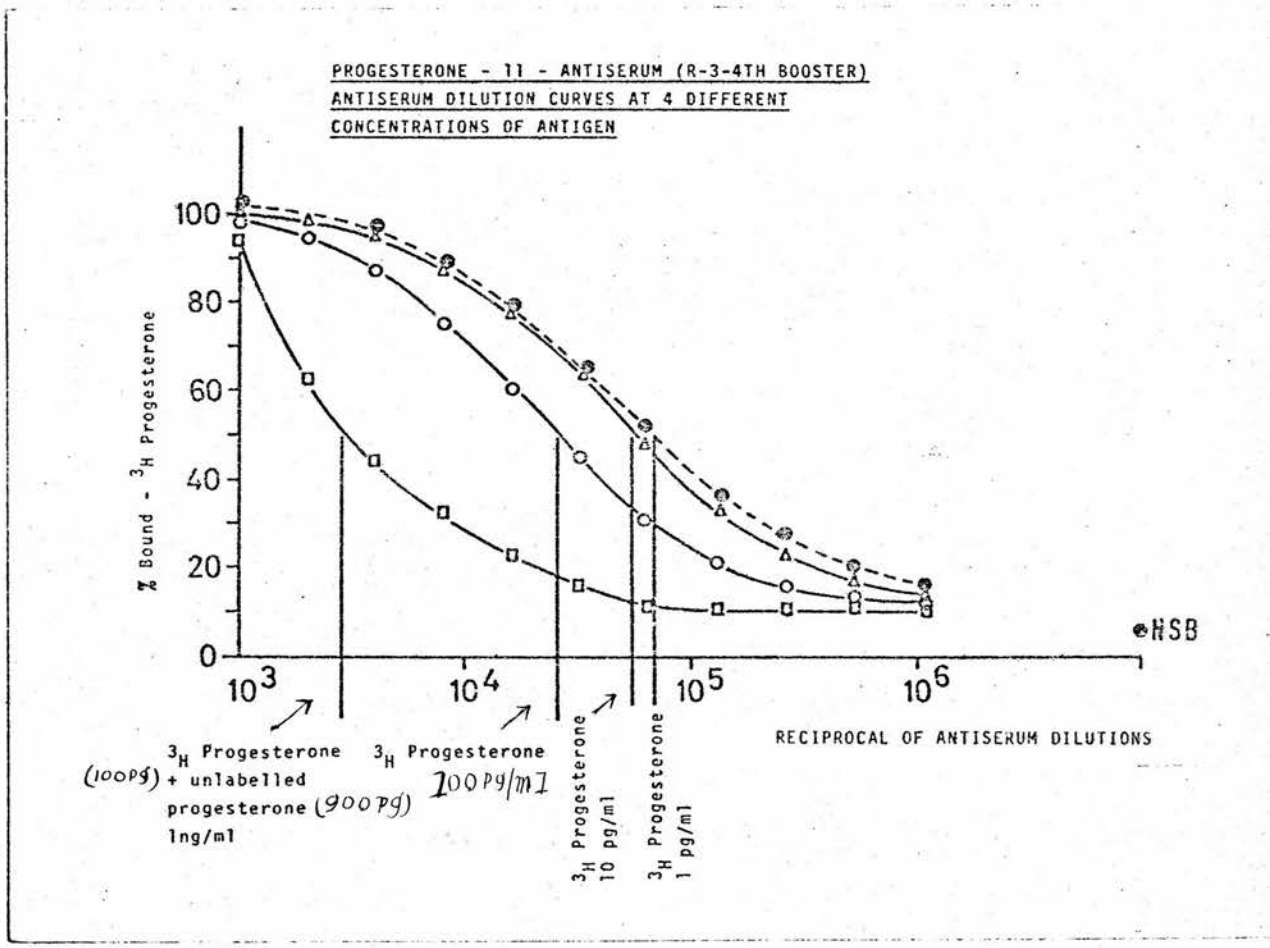
SECTION 3

Results and Discussion

3. Titre and sensitivity of antisera.

The titre of an antiserum is an arbitary term which is used to indicate the amount of antiserum that will bind a stated proportion of added antigen under defined experimental conditions. In fig. 8 a series of dilution curves using 1, 10, 100 pg of labelled progesterone and 1 ng of labelled and unlabelled progesterone (900 pg of unlabelled progesterone + 100 pg of [^{3}H] progesterone) have been plotted. Titre could be defined as the dilution of antibody that will bind 50% of any given concentration of the antigen. As shown in the fig.8 the antiserum can clearly distinguish between the concentrations of 100 pg and 1 ng shown by the 10 fold displacement of the curves but fails to make a similar distinction between 10 and 100 pg as the curves using these dilutions are only a little over two dilutions apart. This suggests that the effective sensitivity of the assay using this antiserum lies somewhere between 10 - 100 pg. There is virtually no displacement of the curves between 1 - 10 pg amounts suggesting a limit imposed by the avidity of the antiserum for the antigen. Titre can thus also be defined as that dilution of an antiserum which binds some stated proportion of tracer (e.g. 50%), this proportion being

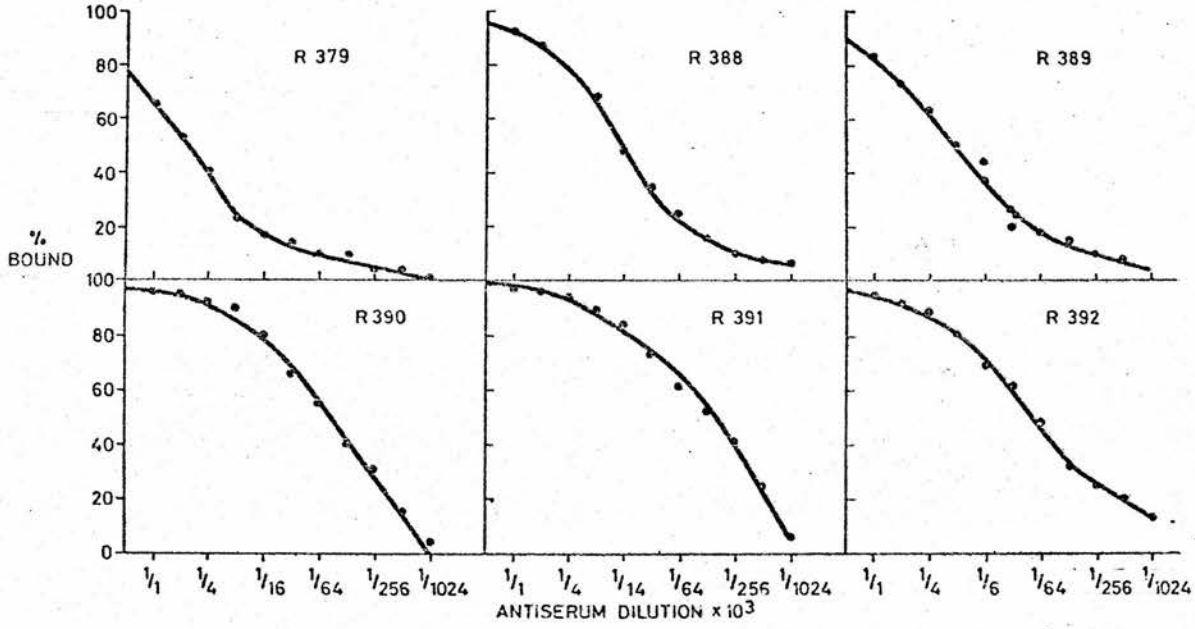
Fig. 8


PROGESTERONE - 11 - ANTISERUM (R-3-4TH BOOSTER)
ANTISERUM DILUTION CURVES AT 4 DIFFERENT
CONCENTRATIONS OF ANTIGEN
% Bound - 3H Progesterone
100
80
60
40
20
0
10^3
10^4
10^5
10^6
NSB
RECIPROCAL OF ANTISERUM DILUTIONS
3H Progesterone (100pg) + unlabelled progesterone 1ng/ml
3H Progesterone (900pg) 100pg/ml
3H Progesterone 10 pg/ml
3H Progesterone 1 pg/ml

unaffected by further decreasing the amount of antigen. Considered in this way the definition of titre is related to the avidity of the antibody for the tracer antigen and it is by this definition that the titres of the antisera have been described in this thesis. Some examples of these dilution curves are shown in fig. 9 .

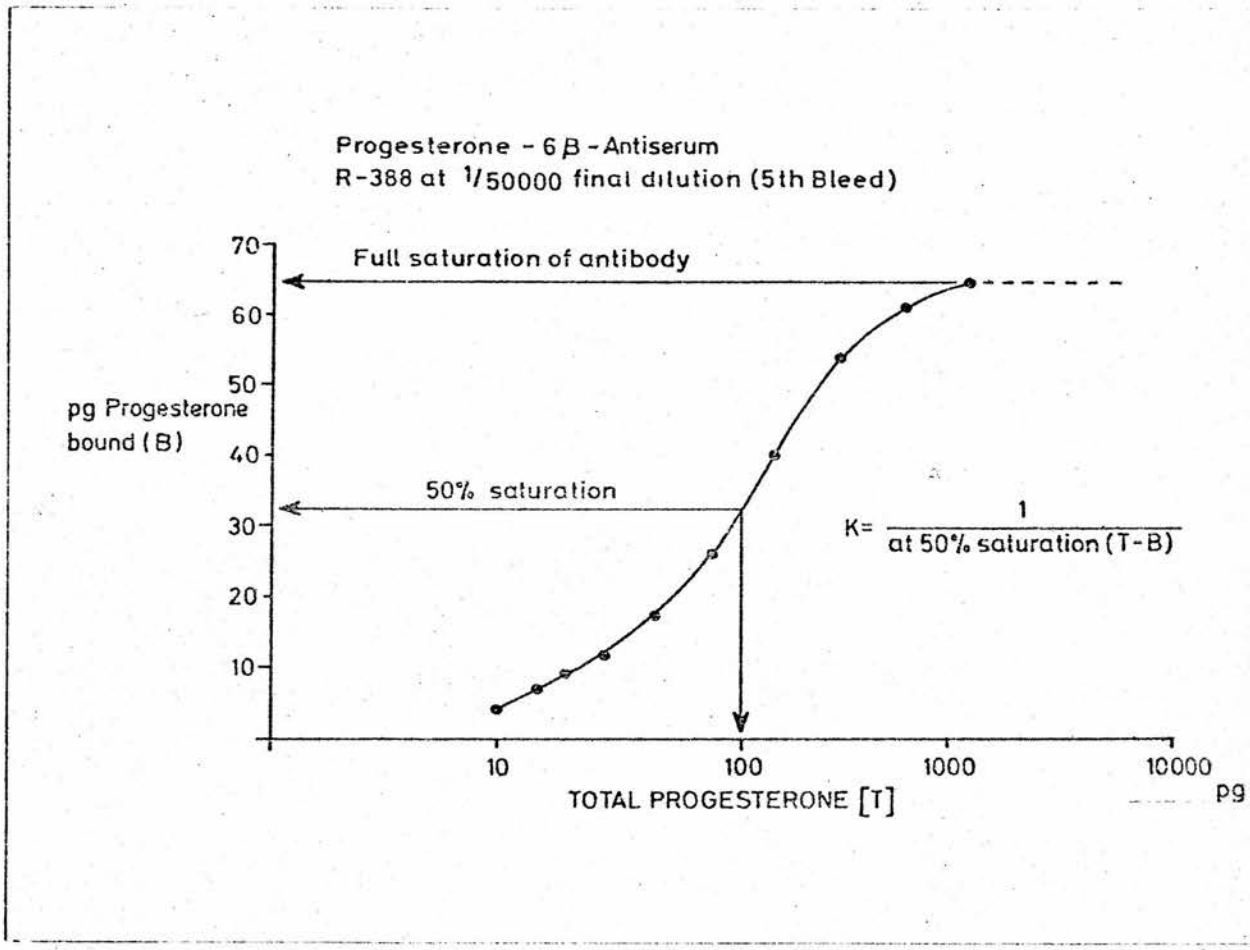
Each antiserum contains multiple or heterogenous populations of antibodies that react with the antigen with different affinities. The effective sensitivity of an antiserum depends on those antibodies that react with the highest energy at a suitable concentration. The affinity constant or K value is a fundamental property of antisera which dictates their sensitivity potential in terms of molarity rather than the mass of the antigen and is of value in comparing the properties of various antisera. However, from the point of view of radioimmunoassays it is arguable whether calculation of the K values of antisera by the method of Scatchard (1949) or by the Michaelis-Menton hyperbola (Odell et al., 1969) yields any other information that cannot be derived from the standard curves which are used to calculate the K values.

Fig. 9


DILUTION CURVES OF PROGESTERONE-6 β-ANTISERA AFTER 1st BOOSTER
(10 pg ^{3}H TRACER ADDED)
% BOUND
R 379
R 388
R 389
R 390
R 391
R 392
100
80
60
40
20
0
1/1 1/4 1/16 1/64 1/256 1/1024
1/1 1/4 1/14 1/64 1/256 1/1024
1/1 1/4 1/6 1/64 1/256 1/1024
ANTISERUM DILUTION x 10^{3}

In the present work the affinity constants of selected antisera have been calculated by a method based on a concept, suggested by Odell et al., (1969), which is similar to that used for the determination of the Michaelis constants of enzymes. In this method a graph of bound to total progesterone is plotted (fig.10) and the concentration of free progesterone at 50% saturation of the antibody is the reciprocal (1/K) of the affinity constant K. The K values so determined depend on the concentration of antibody because with a higher concentration of antiserum a less sensitive standard curve will be obtained which would alter the ratio of bound to total progesterone. The affinity constants as calculated by the method of Scatchard are also concentration dependent (Rosenberg and Notkins 1974). Thus the K values, calculated by either of the methods are dependent on the experimental concentration of the antisera. Therefore to obtain the true K value of an antiserum the experiment should be carried out with the antiserum at an appropriately high dilution for only then will it give information on sensitivity potential.

Fig. 10


Progesterone - 6 β - Antiserum
R-388 at 1/50000 final dilution (5th Bleed)
Full saturation of antibody
50% saturation
pg Progesterone bound (B)
70
60
50
40
30
20
10
K= 1 / at 50% saturation (T-B)
10
100
1000
10000
TOTAL PROGESTERONE [T]
pg

Using the optimum dilutions of antisera and trace quantities of tracer (10 pg) the K values (affinity constants) of three 11α-hydroxyprogesterone-BSA antisera were 3.15×10^9, 3.77×10^9 (R1 and R3, 4th booster respectively) and 3.92×10^9 (R6, 3rd booster) ℓ/M. The affinity constants of the three selected 6β-hydroxyprogesterone-BSA antisera were 2.92×10^9, 3.55×10^9 (R 388 and R 389, 5th booster) and 3.92×10^9 (R 390, 3rd booster) ℓ/M.

There is no unanimity on the definition of sensitivity among theoreticians. Yalow and Berson (1969) have defined the sensitivity and precision in terms of the slope of the dose-response curve while Ekins (1969) is of the opinion that both sensitivity and precision depend not only on the slope but also on the errors involved in the determination of points on the response curve. According to Ekins (1970) while precision is the ability of an assay system to distinguish between two hormone concentrations at any point on the response curve the term sensitivity may be used to restrict one of these points to a zero amount or concentration. According to this concept sensitivity or detection limit is defined as the minimum amount of hormone that can be

measured with acceptable precision. Defined as such it is the lowest concentration of unlabelled antigen which results in a significant change in the response variable, that is, a change equivalent to the standard deviation of the replicates of the zero standards multiplied by a "significant" value of the Students t (approximately 2) (Hunter and Greenwood 1964, Borth 1970, Feldman and Rodbard 1971). However, determination of sensitivity in this manner requires an accumulation of data gathered from several experiments for an accurate estimation of the standard deviation and this is not always practicable in routine assay work. An alternate approach has been chosen in the present work whereby antisera sensitivities have been compared by measuring responses at a constant factor of a 10% fall in % binding of the tracer. Measured in this manner sensitivities have been arbitarily defined as that concentration of standard progesterone (in units/ml incubate) which depresses the binding of labelled progesterone by 10% (Hunter 1971 and 1973). This expedient assumes that the errors involved are unrelated to the individual antisera and is valid for comparative

purposes in systems in which the conditions (e.g. mass of tracer, binding of tracer in the absence of added unlabelled antigen) are identical in curves being compared.

Effect of booster injection and time on the titre and sensitivity of antisera:

The effects of booster injections and time on the titre and sensitivity of antisera is shown in figs.11,12 and 13. Fig. 11 and 13 shows the immunization schedule of rabbits immunised with 11α-hydroxyprogesterone-BSA and 6β-hydroxyprogesterone-BSA conjugates over a 30 and 34 week period respectively and fig. 12 shows the immunization schedule over longer periods of 46 weeks (R3) and 120 weeks (R1) in rabbits immunized with 11α-hydroxyprogesterone-BSA conjugates.

In the 11α-hydroxyprogesterone-BSA immunized groups of rabbits maximum titres were obtained after 14 - 18 weeks. The titres in 6β-hydroxyprogesterone-BSA immunized groups were still rising when the immunization was stopped at 34 weeks, the significant feature here being that the titres did not drop whether or not the

Fig. 11

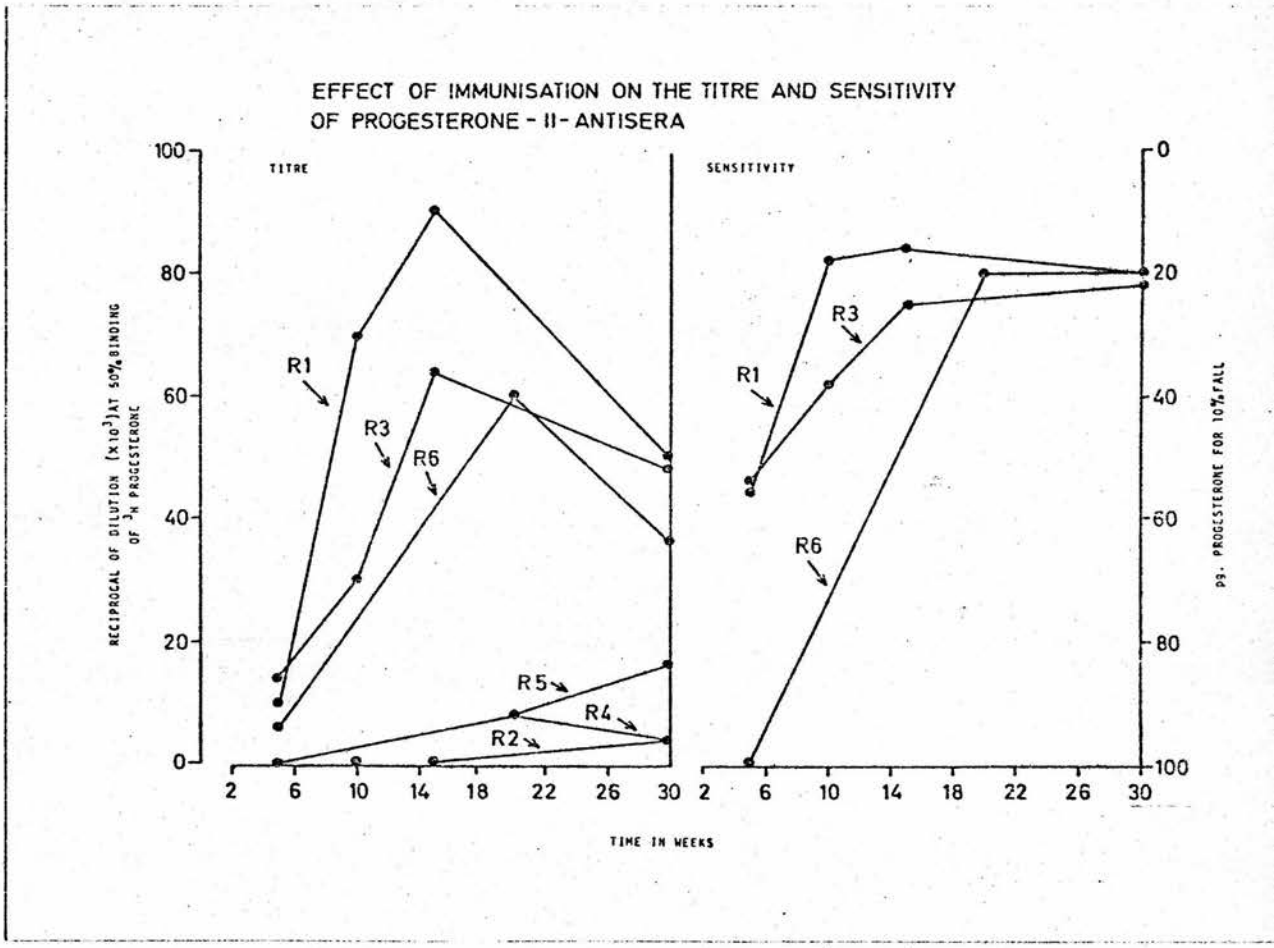

EFFECT OF IMMUNISATION ON THE TITRE AND SENSITIVITY
OF PROGESTERONE - 11- ANTISERA
TITRE
SENSITIVITY
RECIPROCAL OF DILUTION (X10³) AT 50% BINDING OF ³H PROGESTERONE
pg. PROGESTERONE FOR 10% FALL
TIME IN WEEKS
R1
R2
R3
R4
R5
R6
100
80
60
40
20
0
2
6
10
14
18
22
26
30

Fig. 12

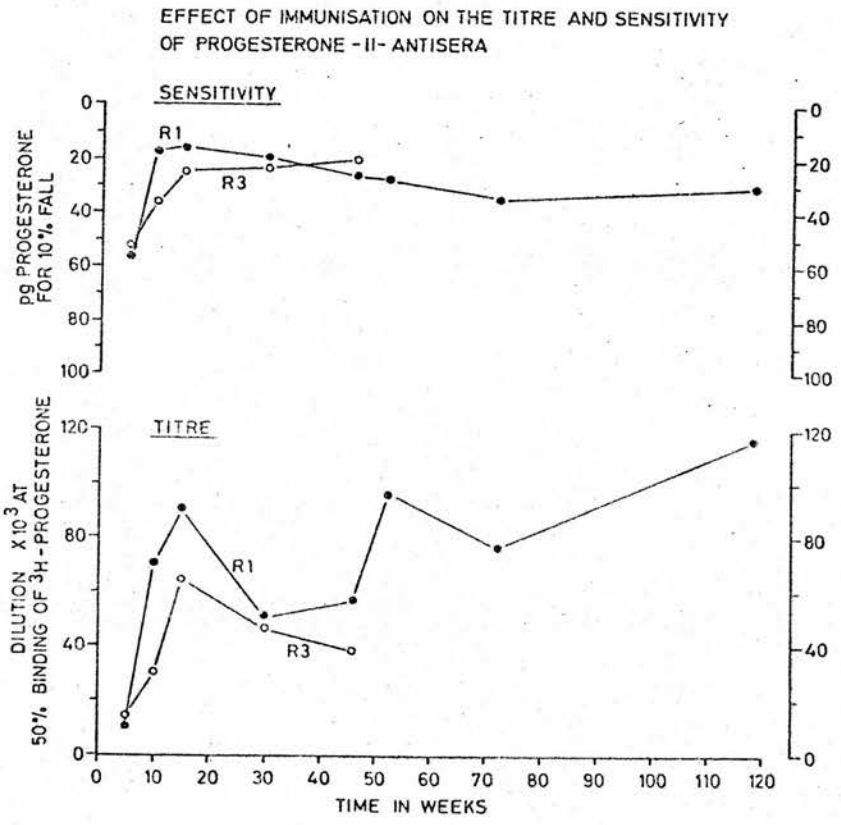
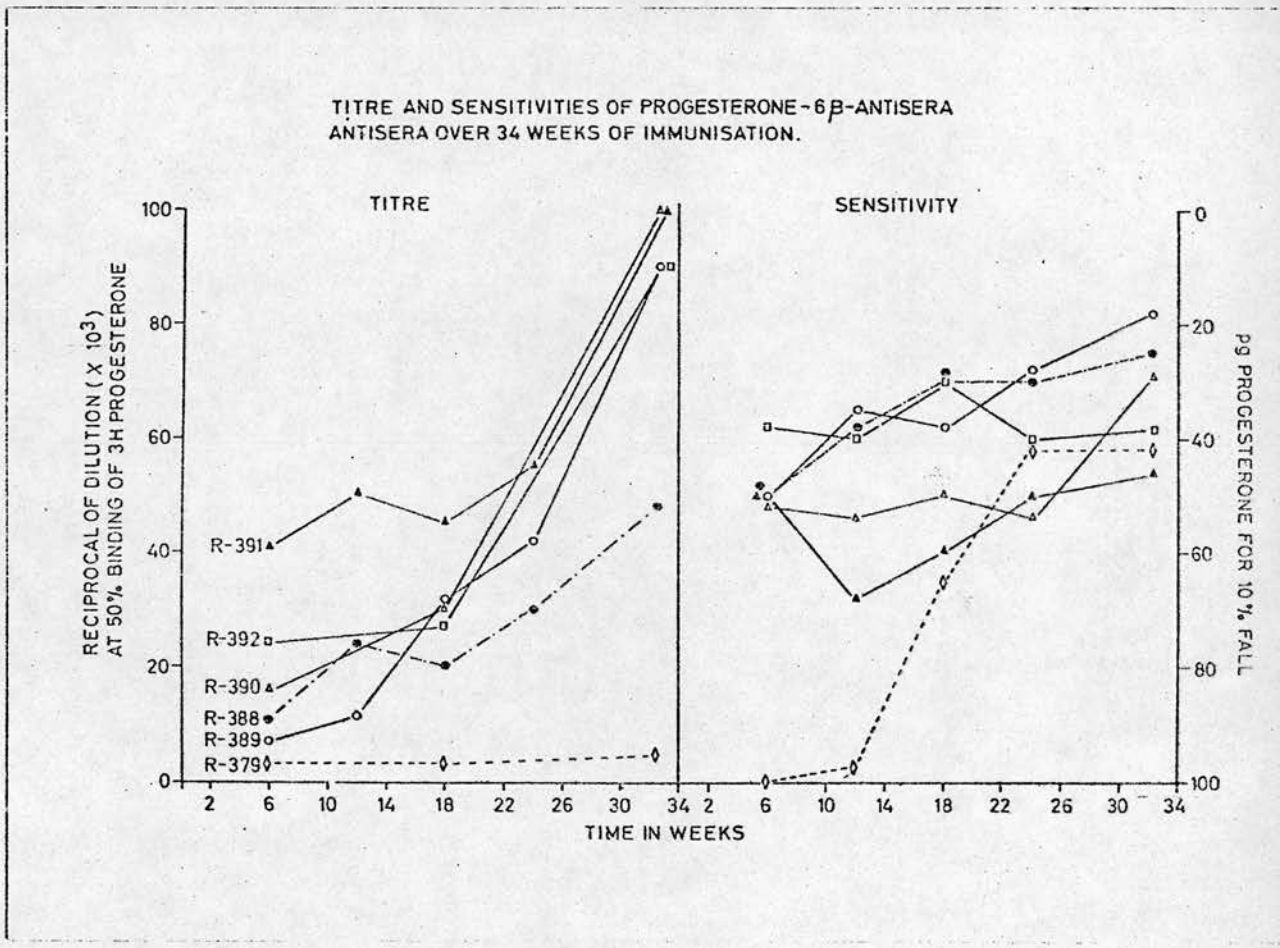

EFFECT OF IMMUNISATION ON THE TITRE AND SENSITIVITY
OF PROGESTERONE - II- ANTISERA
SENSITIVITY
pg PROGESTERONE FOR 10% FALL
R1
R3
TITRE
DILUTION X 10³ AT 50% BINDING OF ³H-PROGESTERONE
R1
R3
TIME IN WEEKS

Fig. 13



All the rabbits were bled once every six weeks.

1. R388, 389 and 391 were boosted once every six weeks.

2. R379, 390 and 392 were boosted once every twelve weeks.

rabbits were boosted. Thus the titres of antisera from rabbits - 379, 390 and 392 at weeks 12 and 24, when they were not boosted, were comparable to the titres from the other rabbits which were regularly boosted.

On the whole although titres of antisera tended to improve on boosting the responses obtained were very variable over the time period of the study. These observations are in agreement with those of Thorneycroft et al. (1970) and Hanning et al., (1972) who obtained variable titres in antisera from sheep immunized with oestradiol-17 β conjugate over 10 months and aldosterone-BSA conjugate over 262 days respectively. Abraham (1974) found that titres reached a plateau in 6 - 8 months.

With respect to the effects of booster injections and time on the sensitivities of antisera it was observed that maximum sensitivities were obtained in the first $3\frac{1}{2}$ - 4 months of immunization and remained more or less constant thereafter throughout the immunization schedule. This is in contrast to the studies of Abraham (1974) who found a consistent rise in the sensitivities of antisera over a 13 month period.

In summary, from the small number of animals studied, it appears that the effect of booster injections and time on the titres of antisera are variable whereas maximum sensitivities once attained are likely to remain unaltered. Secondly, the effects of long rests between booster injections does not confer an additional advantage in terms of titre or sensitivity of antisera over that of the 5 - 6 weekly injections. These observations are in agreement with those of Lader et al., (1973) who raised antisera to insulin in guinea pigs by subcutaneous injections and found comparable results in terms of titre and affinity between the groups of animals which were injected monthly and those rested for 4 months at different times during the immunization procedure.

SECTION 3

Results and Discussion

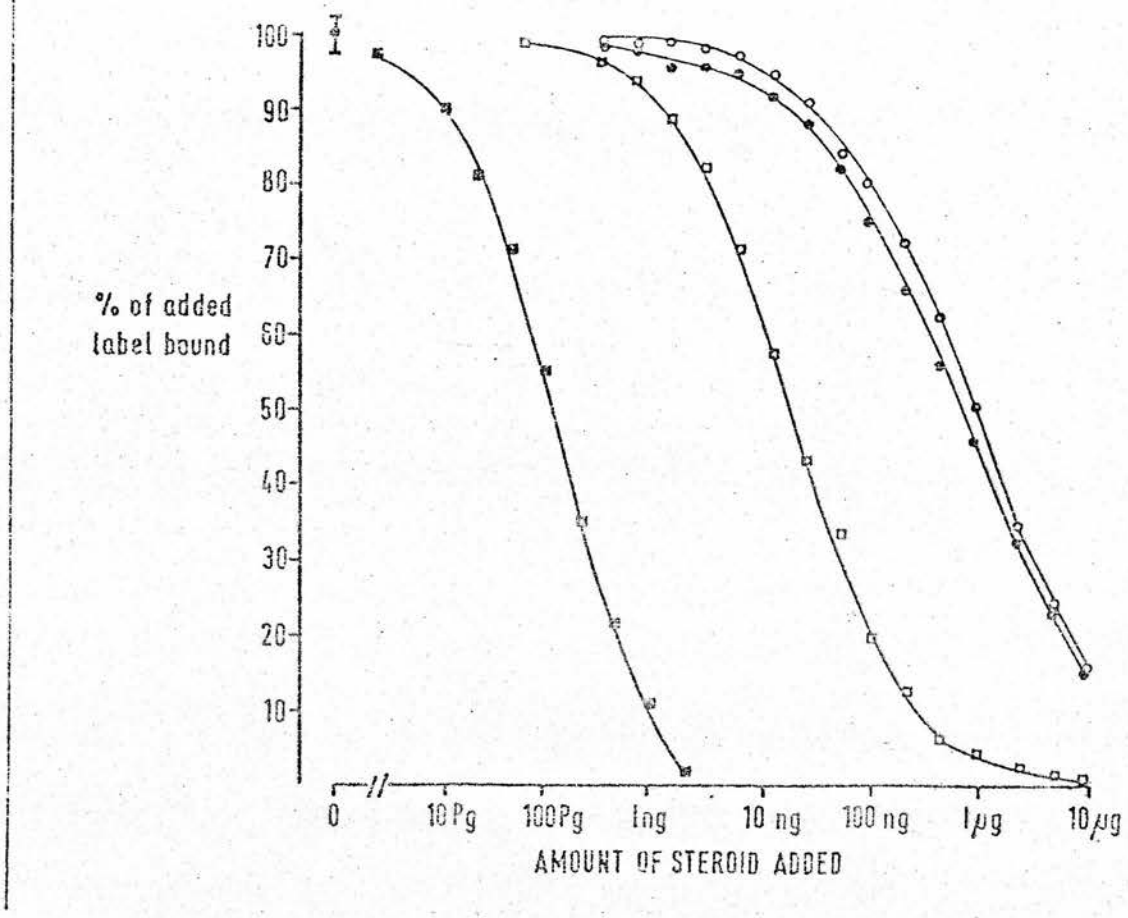
4. Specificity of progesterone antisera.

Cross reactions have been defined as the reactions of a given antibody population with more than one antigen (Little and Eisen 1969). In the present work the antisera have been raised to the 11α-hydroxyprogesterone-bovine serum albumin and 6β-hydroxyprogesterone-bovine serum albumin conjugates. The 11α-position is well removed from the functional groups of progesterone and being α-in configuration, it is below the mean planar structure of the progesterone molecule. Coupling to protein at this position through a hemisuccinate group was likely to minimise masking of the steroid by the protein. Coupling at the 6β-position had the disadvantage that the protein moiety in the conjugate was on the same side as the progesterone molecule and in spite of the relatively longer hemisuccinate link some masking of the steroid moiety in the conjugate was possible. Secondly, because the 6β-position is near the Δ^4-3 oxo structure, antisera raised to conjugates linked at the 6β-position were likely to give higher cross reactions with those steroids or metabolites that structurally differed from progesterone at or near the 6- position. In this respect the selection of 6α or 7α as link positions could have been more effective.

Progesterone antisera have now been raised against conjugates linked at the 6α-position (Niswender 1973, Jones and Mason 1974) and the 7α-position (Bauminger et al. 1973). The compounds 6α-hydroxyprogesterone and 7α-hydroxyprogesterone were not commercially available when the present work was started. However, judging by the varied specificities of these antisera to steroids differing in structure from progesterone at or near the 6α- or 7α-positions (Pregn-5-en-3β-ol-20-one, , 5α-pregnane-3,20-dione), these sites do not appear to confer any additional advantage over the 6 β-position.

Extensive specificity studies have been carried out only on those antisera which have given the best sensitivities and titres. During the specificity experiments, the dose response curves of steroids that cross react in the assay system were found to be non-parallel to those of progesterone itself. Changes in the structure of the cross-reacting steroids resulted not only in the expected lateral displacement of such curves but also in progressively diminishing slopes as their relative cross-reactivity decreased. This is illustrated in fig. 14 for 3 compounds 17-hydroxyprogesterone, androst-4-ene-3,17-dione and cortisol. For example,

Fig. 14



Detailed dose-response curves for progesterone (■) and three cross-reacting steroids, 17-hydroxyprogesterone (□), androst-4-ene-3,17-dione (●) and cortisol (○)

The responses are plotted with the binding of [^{3}H] progesterone tracer (10pg in 1 ml final incubation volume) in the absence of unlabelled progesterone as 100% bound [first point (●) ± S.D. in the Figure] and non-specific binding controls as 0% bound. The response curves for the cross-reacting compounds are not strictly parallel to that of progesterone itself but show diminishing slopes as the degree of cross-reactivity falls. Each point represents the mean of duplicate tubes. About 70% of the tracer was bound in the absence of unlabelled steroid.

cortisol gave relative potencies of 0.038, 0.018 and 0.011% while the figures for 17-hydroxyprogesterone were 1.0, 0.83 and 0.61 respectively at 20, 50 and 80% inhibition of tracer binding (Progesterone 100). The differences in relative potencies which emerge by accepting the responses at 20, 50 or 80% inhibition of tracer binding on the cross-reaction curves of the majority of steroids are small and detailed studies as well as high precision are necessary to show the small deviations from parallelism.

The observations in the present studies are in agreement with the theoretically predicted studies of Ekins (1968) and Rodbard (1970). According to these workers the relative potency of the two compounds varies as a function of the extent to which the tracer ligand is bound and parallel dose response curves between two compounds are obtained only if they react with an antibody with identical affinities.

The degree of cross reactivity of a given compound cannot thus be fully represented by a single figure expressing its relative potency since this differs

throughout the response-curve. The general practice of expressing relative potencies in terms of single ratios from the intercept of their dose-response at 50% inhibition (Abraham 1969) of tracer binding is not strictly valid unless the responses are parallel, and where they are not its use implies a specificity which is better than can be achieved in practice at the upper half of the response curve but which is less than can be expected in its lower half. However, as these differences in relative potencies at different points on the response curves are small the general specificities of the antisera may be represented at least in relative terms at the 50% inhibition point and they have been so calculated in the present studies and are set out in Table 9 . However these values should not be taken to be the true cross-reactions in the assay and should be separately checked. This is especially necessary in situations where progesterone secretion is low (in follicular-phase; or in men) and the plasma responses in the assay are limited to the upper part of the response curve because in such situations steroids like cortisol or 17 hydroxyprogesterone can introduce non-specificity to the assay endpoint.

Response curves over the range 1 pg - 10μg were run on various cross-reacting steroids. 25 compounds have been tested with antiserum R1 which was finally selected for the progesterone assay. Antisera from rabbits R3 and R6 were tested with 20 compounds and the selected antisera from the 6 β-hydroxyprogesterone-BSA immunized rabbits (R388, R389 and R390) were tested with 22 compounds.

Table 9 sets out the specificities of three 11α-hydroxyprogesterone-BSA immunized and the three 6 β-hydroxyprogesterone-BSA antisera. The data is derived from several experiments and the conditions used have been set out in detail in Table 9 and were identical with those used in the routine assay.

The data in Table 9 shows that the specificities of the three 11α-hydroxyprogesterone-BSA antisera were remarkably similar to one another; those of 6 β-hydroxyprogesterone-BSA group of antisera although different from the 11α-hydroxyprogesterone-BSA immunized group were again remarkably similar to one another.

Table 9

Specificity studies on rabbit antisera to 11α-hydroxyprogesterone-bovine serum albumin and 6β-hydroxyprogesterone-bovine serum albumin conjugates

Steroids were dissolved in the assay diluent. Tubes with a maximum concentration of 10 μg/ml contained 1% (v/v) ethanol and these were serially diluted down to a concentration of 1 pg/ml. Incubation was for 2 h and separation was achieved by the double-antibody method. Responses were calculated at 50% inhibition of tracer binding except for cholesterol, whose maximum displacement (10% at 10 μg/ml) was used.

	Specificities of rabbit antisera to 11α-hydroxyprogesterone-bovine serum albumin (% cross-reaction)			Specificities of rabbit antisera to 6β-hydroxyprogesterone-bovine serum albumin (% cross-reaction)		
	R.1 4th Booster	R.3 4th Booster	R.6 3rd Booster	R.388 5th Booster	R.389 5th Booster	R.390 3rd Booster
Progesterone	100	100	100	100	100	100
11α-Hydroxyprogesterone	31	33	36	9.3	9.1	12.0
11α-Hydroxyprogesterone-hemisuccinate	78	69	49	-	-	-
6β-Hydroxyprogesterone-hemisuccinate	-	-	-	69.0	83.0	82.0
11β-Hydroxyprogesterone	12	4	14	1.7	0.8	3.3
11-Oxoprogesterone	15	12	16	4.0	5.3	3.3
6β-Hydroxyprogesterone	0.8	0.9	1.0	56.0	20.0	28.0
20α-Hydroxypregn-4-en-3-one	1.0	1.0	2.4	1.8	0.91	1.1
20β-Hydroxypregn-4-en-3-one	0.05	0.1	0.1	0.1	0.05	0.04
5α-Pregnane-3,20-dione	13	7.5	17	68.0	55.0	57.0
5β-Pregnane-3,20-dione	7	17	7	18.0	14.0	20.0
17-Hydroxypregesterone	1.2	2.4	1.4	0.2	1.25	0.53
3α-Hydroxy-5β-pregnan-20-one	0.2	0.2	0.4	0.5	0.15	0.4
5α-Pregnane-3α-20α-diol	0.0001	0.002	0.0003	-	-	-
5α-Pregnane-3β-20α-diol	0.003	0.03	0.003	-	-	-
5β-Pregnane-3α-20α-diol (pregnanediol)	0.008	0.035	0.05	0.014	0.002	0.043
3β-Hydroxy-pregn-5-en-20-one	0.13	0.11	0.05	8.0	1.0	5.3
3β,17-Hydroxypregn-5-en-20-one (17-Hydroxypregnenolone)	0.02	-	-	0.01	0.003	0.01
11-Deoxycorticosterone	0.9	4.0	1.9	0.7	1.43	1.44
11-Deoxycortisol (compound S)	0.15	0.07	0.16	0.47	0.45	0.53
Cortisol	0.02	0.03	0.03	0.02	0.002	0.001
Corticosterone	0.43	-	-	0.025	0.005	0.06
Cortisone	0.02	-	-	0.01	0.003	0.01
Androst-4-ene-3,17-dione (androstenedione)	0.02	0.09	0.03	0.09	0.27	0.08
3β-Hydroxyandrost-5-en-17-one (dehydroepiandrosterone)	0.004	-	-	0.025	0.01	0.01
Cholesterol	0.0004	0.0008	0.0007	0.005	0.002	0.002
Cholic acid [3α,7α,12α-trihydroxy cholanic acid]	Not detected	-	-	-	-	-

The specificities of the 11α-hydroxyprogesterone-BSA group of antisera was such that divergencies from the structure of progesterone which were distal to the conjugation site were equally well seen whether they were present in the A or the D ring or on the C-21 side chain. Generally a single change in A ring (pregnenolone), B ring (6 β-hydroxyprogesterone) or D ring (17-hydroxyprogesterone) produced an approximately one-hundred fold loss in potency. Multiple changes in the cross reacting steroid appeared to be essentially additive in their effect. As expected, antisera to the 11α-hydroxyprogesterone-BSA conjugate showed relatively little ability to detect changes in substituents at the 11- position. The overall specificity of these antisera then, is such that substances likely to be present in blood and extractable with progesterone by non-polar solvents are so present at levels which would not be expected to interfere in the assay for progesterone.

The antisera to 6 β-hydroxyprogesterone-BSA did not detect well substituents at the 6- position and changes close to this position. For example, these antisera did not detect well the loss of the C-4 double

bond as in the case of 5α and 5β pregnane -3, 20-dione or a shift in the double bond to the C-5 position and change of 3 oxo to 3-OH as in the case of pregnenolone. In all other respects their cross reactions are similar to those of 11α-hydroxyprogesterone-BSA immunized group of antisera. Assays based upon these antisera would therefore be more vulnerable to interference from pregnenolone and metabolites of progesterone which embrace changes near or at the 6-position. Conversely, the use of a conjugate involving 6- position has not conferred a proportionate improvement in specificity with respect to progesterone derivatives which have small changes at the 11- position. The overall picture therefore leads to a clear though not overwhelming preference for the antisera to 11α-hydroxyprogesterone-BSA.

Effect of Time and Booster Injections on the Specificity of Antisera:

The effect of time and booster injections on the specificity of antisera was studied on the selected 11α-hydroxyprogesterone-BSA antisera after the 2nd booster. Antisera from Rabbit 1 was studied using 7 bleeds up to the 8th booster over a period of 120 weeks.

Antisera from rabbit R3 was studied using 4 bleeds over 46 weeks and that from rabbit R6 was studied with 2 bleeds over 30 weeks. Cross reaction studies of these different samples of antisera were performed with 8 selected steroids. The results are described in Table10 for rabbit R1 and in Table11 for rabbits R3 and R6.

The results show that the specificities of all antisera for compounds pregnenolone, cortisol and 11-deoxy cortisol remained essentially unaltered in the three rabbits through the periods covered. Only minor variations were seen in the specificities for 11α-hydroxyprogesterone, 11-deoxycorticosterone and cholesterol. The specificity for 11 β-hydroxyprogesterone decreased nearly three-fold after the 2nd booster but then remained unchanged in all the three rabbits in the subsequent immunizations. In the case of 17 hydroxy-progesterone there was a minor decrease in specificity after the 3rd booster, specificity then remaining unaltered following all of the subsequent boosters.

Table 10 EFFECT OF TIME AND BOOSTER INJECTIONS ON RABBIT R1 ANTISERA

	COMPOUNDS	Number of Boosters						
		% Cross Reactions						
		2nd	3rd	4th	5th	6th	7th	8th
	Progesterone	100	100	100	100	100	100	100
1	11∝ -Hydroxy progesterone	31.0	29.5	31.0	29.0	41.0	35.0	35.0
2	11β -Hydroxy progesterone	4.75	20.0	12.0	12.0	14.0	12.0	12.5
3	17-Hydroxy progesterone	0.3	0.3	1.0	1.0	0.7	1.0	1.0
4	11-Deoxycorticosterone (Doc)	0.9	1.7	0.9	1.0	0.9	1.0	1.0
5	11-Deoxy cortisol (compd. S)	0.06	0.10	0.10	0.20	0.30	0.15	0.20
6	Cortisol	0.007	0.01	0.02	0.01	0.01	0.008	0.01
7	Pregnenolone	0.20	0.10	0.10	0.20	0.07	0.09	0.09
8	Cholesterol	0.0007	0.0009	0.0009	0.002	0.003	0.004	0.001
9	Androst-4-ene-3, 17-dione	-	-	0.02	0.03	0.017	0.02	0.02
10	20∝ -Hydroxypregn-4-en-3-one	-	-	1.00	0.90	0.60	1.00	1.00
11	20 β-Hydroxypregn-4-en-3-one	-	-	0.055	0.05	0.04	0.05	0.05
12	5∝ -pregnane-3, 20-dione	-	-	13.0	12.0	12.0	23.0	23.0
13	5 β-pregnane-3, 20-dione	-	-	7.0	7.0	5.0	5.0	5.0

Table 11 EFFECT OF TIME AND BOOSTER INJECTIONS ON THE SPECIFICITY OF RABBIT R3 AND R6 ANTISERA

	COMPOUNDS	Number of Boosters					
		% Cross Reactions					
		R3 Antisera				R6 Antisera	
		2nd	3rd	4th	5th	2nd	3rd
	Progesterone	100	100	100	100	100	100
1	11α-Hydroxy progesterone	26.0	33.0	31.0	32.0	29.0	36.0
2	11 β-Hydroxy progesterone	5.0	4.3	12.00	11.0	8.0	14.0
3	17-Hydroxy progesterone	0.20	0.20	2.0	0.90	1.0	1.0
4	11-Deoxycorticosterone 11-Doc	0.60	2.0	4.0	2.0	0.90	2.0
5	11-Deoxycortisol (compd S)	0.02	0.04	0.07	0.20	0.09	0.20
6	Cortisol (F)	0.009	0.009	0.03	0.004	0.01	0.034
7	Pregnenolone	0.03	0.10	0.10	0.05	0.10	0.05
8	Cholesterol	0.001	0.004	0.0008	0.002	0.0003	0.0007

The overall figures for cross-reactions of various steroids show that the specificities of antisera have generally remained unaltered over successive boosters. However, the number of animals studied is too small to make generalizations on the effect of successive booster injections on the specificity of antisera, and there are no comparable studies on the stability of antiserum specificity with time in the steroid radio immunoassay literature. Odell et al. (1969) while studying the effect of immunization on the specificity of antisera to certain protein hormones found that the specificities of antisera to HTSH and HCG were retained or improved with increasing immunizations while that of antisera to HFSH decreased after five immunizations. Abraham (1974) has suggested that specificity of antisera (which were raised in ewes) improves in the first 6 - 8 months with respect to steroids differing from the immunogen at the site of linkage and that this period is then followed by decreased specificity, whereas the specificity of the antisera for steroids differing from the immunogen at the distal position of the molecule increases with the duration of immunization up to 12 months. For example, the specificity of antisera to oestradiol-17-BSA with respect to oestrone and of

antisera to testosterone-3-BSA to 5α-androstane 3 β-17 β-diol improved 2 - 3 fold in the first 6 - 8 months and then decreased 4 - 5 fold afterwards over a 12 month period. The specificity of testosterone-3-BSA with respect to androstenedione (differing in structure at ring D - distal to the link) continued to improve (3 - 4 folds) up to 9 months (Abraham 1974). The author has however warned against extrapolation of these studies on antisera raised in ewes to other animal species.

In the present studies the above specificity trend has not been noticed. Firstly, no steady improvement in specificity of 11α-hydroxyprogesterone-BSA antisera with respect to 11 β-hydroxyprogesterone (the steroid differing at the site of linkage) or any steroid in the first 6 - 8 months was observed. Further, there was no deterioration in specificity after 6 - 8 months as the specificity of R1 and R3 antisera to 11 β-hydroxyprogesterone remained essentially unaltered over a period of 3.5 to 27 months (2nd booster - 8th booster) and from 7 months to 11 months (4th booster - 5th booster) respectively. Secondly, specificity of the 11α-hydroxyprogesterone antisera with respect to steroids

such as cortisol, pregnenolone or androst-4-en- 3,17-dione (differing from the immunogen at either of the distal ends of the molecule) has remained essentially unaltered over the time period of the immunization schedule.

Although the present specificity studies were performed with a small number of animals they do suggest that once the production of a specific antiserum is evoked this specificity is likely to be retained following further booster injections. However, this stability cannot be taken for granted and specificity should clearly be checked after every booster.

SECTION 3

Results and Discussion

5. Progesterone-Assay.

(a) Progesterone-like activity in urine.

(b) Behaviour of unextracted plasma in the assay.

Extraction of progesterone from plasma by petroleum ether:

The choice of light petroleum AnalaR grade from BDH or H&W, B.P. 40 - 60°C) as the solvent for extracting progesterone from plasma was based on the following: non-polarity, low boiling range, low cost and easy availability. The main disadvantage is that each batch has to be checked for its suitability for the assay. For this purpose the standard purification procedure for hydrocarbons has been slightly modified and the details of this procedure are given in the methods section.

The main problem when using light petroleum as the extraction solvent was the variability of extraction rates (70 - 90%) of the radioactive progesterone from plasma. The addition of ethanol to plasma prior to the extraction significantly reduced this variability, and the recoveries of progesterone were markedly improved. The results of these extraction recoveries after the addition of ethanol 10% v + v (1 vol. ethanol + 10 vol. plasma) are shown in Table 12 . A single extraction gave a mean of 92.4 ± 1.2 (SD) for 50µl - 200µl plasma using 2 ml solvent and extraction was quantitative if a second 10 vol. (2 ml.)

Table 12 Extraction of [^{3}H] progesterone from plasma by light petroleum

Plasma was equilibrated with [^{3}H] progesterone for 15 min. at 20°C. Ethanol was then added to the plasma (10 vol. plasma + vol. ethanol) and after a further 15 min. at 20°C the plasma was extracted with light petroleum. Four replicates were used for each group.

Vol. of plasma (µl)	Vol. of light petroleum (ml)	Extraction (%)			
		One extraction		Two extractions	
		Mean	S.D.	Mean	S.D.
50	2.0	93.12	1.24	99.22	1.5
100	2.0	93.45	1.40	99.72	2.0
200	2.0	91.52	1.54	100.3	1.5
500	5.0	91.5	0.7	99.4	0.93
Mean		92.4	1.2	99.7	1.5
500	2.5	79.6	3.5	96.8	1.1

extraction was included. For the larger plasma volume (500 µl), necessarily used when progesterone was to be measured in men or post menopausal women, the single extraction of 1 vol. x 10 vol. gave 91.5 ± 0.7 (SD). For the routine assay therefore ethanol (10% v + v) was added to the plasma. After standing for 15 minutes the ethanolic plasma was extracted with light petroleum, 2 ml of which was used for plasma vols of 200 µl or less and 5 ml for 500 µl aliquots. After extraction the aqueous layer was frozen by standing the rack of tubes in solid CO_2: ethanol, the organic layer decanted into glass tubes 75 x 17 mm, evaporated to dryness and the residue assayed in situ.

Effect of solvent residues on assay:

A series of experiments was carried out in order to examine whether solvent residues produced by the above procedure might distort the standard curve of the assay. Progesterone was added over the range 5 pg - 10 ng in ethanol solution which was then evaporated to dryness and the residue redissolved in water (200 µl). Ethanol

(10 vol. water + 1 vol. ethanol) was added and the progesterone was then extracted into light petroleum (2 ml), the aqueous phase frozen and the organic phase decanted into the assay tubes. After evaporation, these tubes were assayed together with similar standards which were made up by adding the assay reagents (tracer and antiserum) directly into tubes containing the residues from the ethanolic solution of progesterone.

Fig. 15 (a, b, c, d) shows the two different ways in which the solvent residues could distort the dose-response curve of the standards. In each case the curve obtained for the standards in diluent only is shown by open circles, and those run in tubes containing solvent residues are shown by the open triangles. Most batches of solvent yielded curves of the kind shown in Fig. 15 (a). Two factors appeared to be distorting the dose-response curve. In Fig. 15 (b) the light petroleum had been purified as described above, although some batches of solvent gave curves of this kind without purification. The distortions at this end of the curve were generally less severe in solvent blanks than in water blanks. The remaining perturbation which is seen at higher concentrations of progesterone in Fig. 15 (a) was always seen when the

Fig. 15(a)

Effect of solvent residues on the dose-response curve

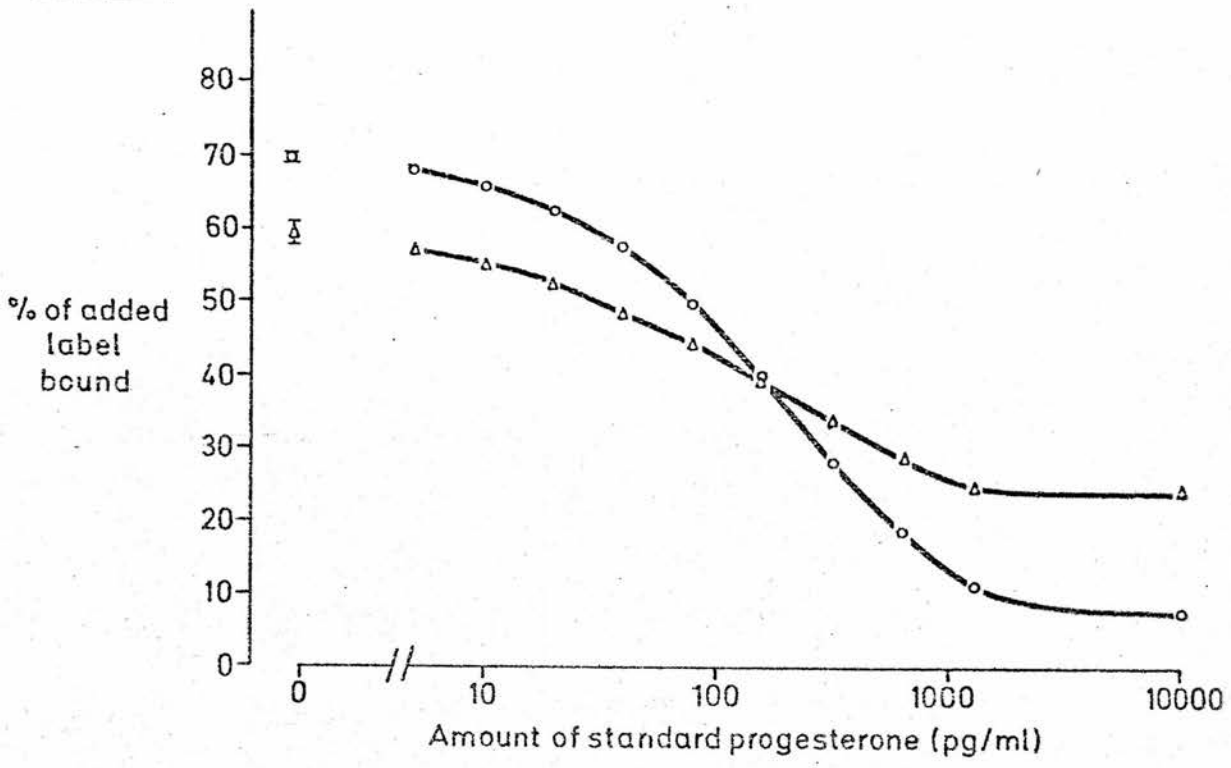
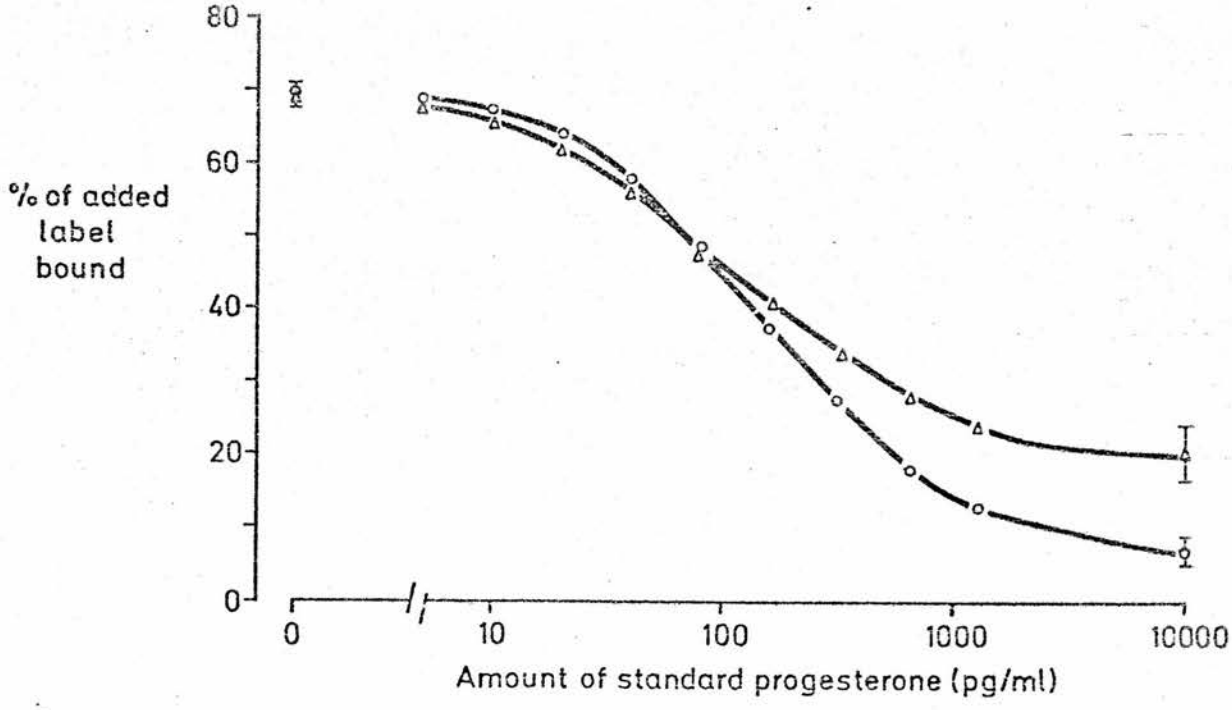


Fig. 15 (b)



The legend for Fig. 15(a) and (b) is described on p. 146(a)

Fig. 15(c)

Effect of solvent residues on the dose-response curve

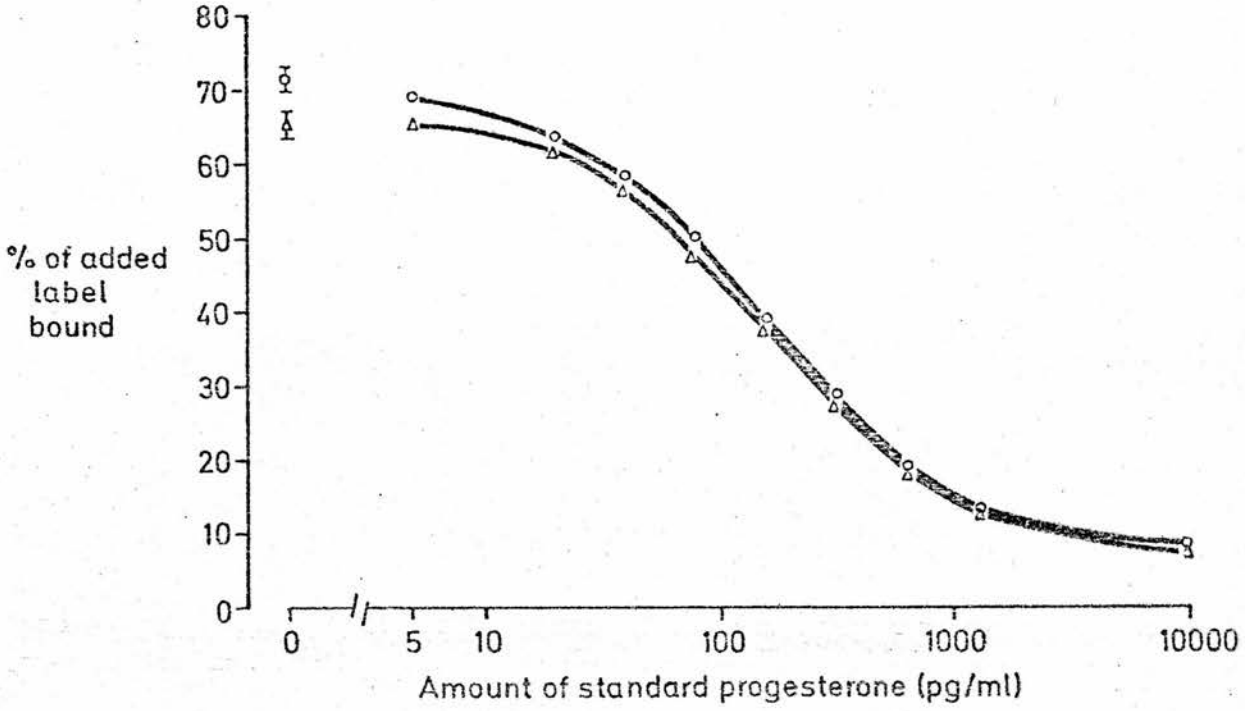
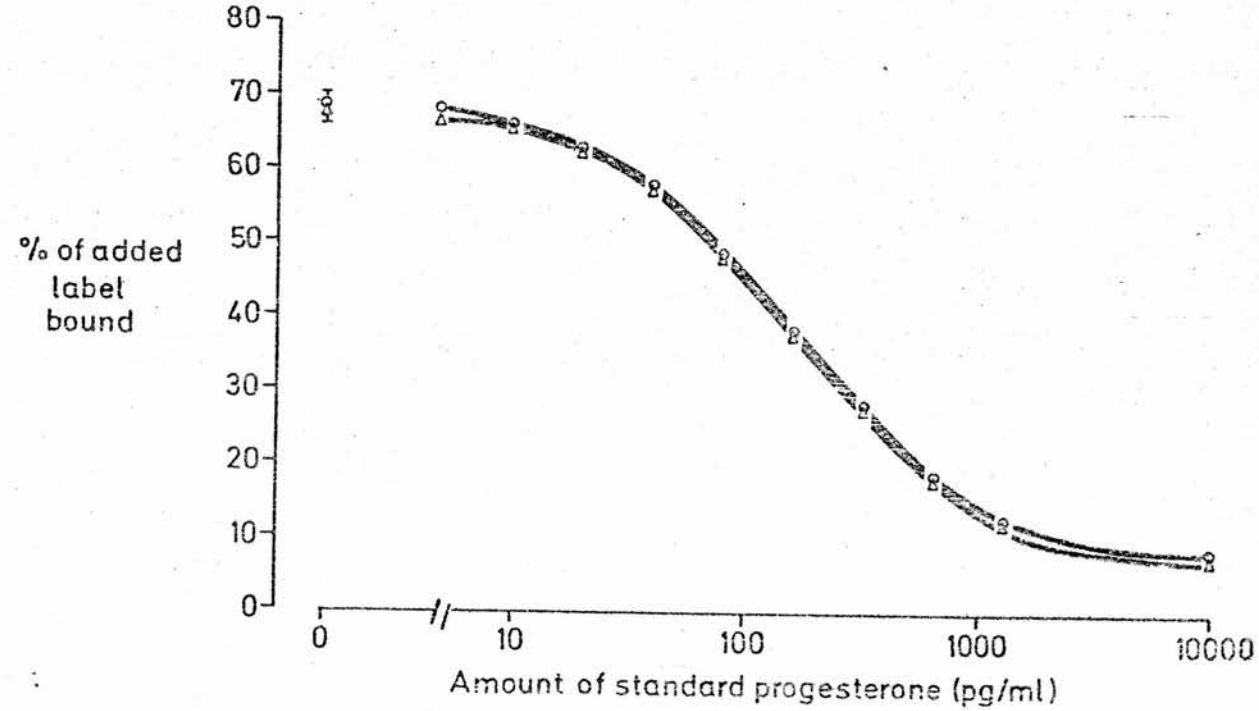


Fig. 15 (d)



The legend for Fig. 15(c) and (d) is described on p. 146(a)

Fig. 15. Effect of solvent residues on the dose-response curve

In each case the curve with open circles represents a standard curve in diluent. The curve with open triangles represents the standard curve from tubes in which the dried progesterone standards were redissolved in water (200µl) and extracted with light petroleum by the standard extraction procedure as described in the text. The first values on each curve represent (± S.D.) the percentage [^{3}H] progesterone tracer (10pg in 1 ml final incubation volume) bound in the absence of unlabelled progesterone. All other values are expressed as the means of duplicates. The four parts to the Figure show the standard curve as (a) water-extracted in impure solvent with drying of tubes at room temperature; (b) water-extracted in purified solvent with drying of tubes at room temperature; (c) water-extracted in incompletely purified solvent but with drying of tubes under air at 60°C; (d) water-extracted in purified solvent with drying of tubes under air at 60°C.

light petroleum was evaporated under a stream of N_2 or air at room temperature. When assay diluent and then [^{3}H] progesterone was added to tubes containing these residues only 70 - 85% of the tracer was removed when the aqueous solution was decanted into counting vials. This was presumably due to the presence of solvent contaminant which dissolved some of the tracer and rendered it unavailable to aqueous dissolution. This effect was only slightly diminished by the solvent purification described under methods (Fig. 15 (b). However, it was completely abolished (Fig. 15 (c) by heating the residue to 60°C for 15 minutes under N_2, or more conveniently by carrying out the entire solvent evaporation process under N_2 or air at 60°C before addition of assay diluent. When purified solvent was used and the evaporation temperature also raised, the curve almost invariably was identical with that obtained in diluent (Fig 15 d). Since distortions caused by solvent residues can, if unrecognized, give rise to both positive and negative artifacts, two standard curves were run in all assays. One standard curve was carried out without extracting the standards, these being added, in ethanol, to the assay tubes, evaporated under nitrogen and dissolved in diluent. In the second standard

curve the standards were added in ethanol, evaporated to dryness, the residues dissolved in 200µl of water and then extracted with light petroleum after the addition of 20µl of ethanol as in the standard extraction procedure. These two standard curves have been superimposable in 7 out of 11 consecutive assays carried out over 13 weeks and using four different batches of solvent. The remaining four assays gave slightly displaced curves (similar to that shown in Fig. 15(C) for the standards based on water blanks and these, rather than the curve for standards in diluent, were used to calculate the results of these assays.

Table 13 shows results for the recovery of unlabelled progesterone added in different amounts over the range 160 pg/ml to 12.8 ng/ml to portions from a pool of male plasma from which different volumes were extracted and assayed. By using a single extraction, recoveries over the whole range of different amounts of progesterone and of different plasma volumes of 91.4 ± 8.6% (S.D.) were obtained, and two such extractions over the plasma volume range 50 - 200µl gave 96.8 ± 6.6% (S.D.). These findings confirm the high and constant

Table 13 Assay recoveries of non-radioactive progesterone added to pooled male plasma

Ethanol (10 vol. plasma + 1 vol. ethanol) was added after equilibration of the plasma samples with dried unlabelled progesterone and extraction with light petroleum performed after another 15 min. Progesterone was assayed as described in the text. In each case four replicates were run. The mean and S.D. show their scatter in each group.

Progesterone added	Vol. of plasma	Vol. of light petroleum	Recoveries (%)			
			One extraction		Two extractions	
	(μl)	(ml)	Mean	S.D.	Mean	S.D.
80 pg	100	2	87.5	8.7	-	-
	200	2	89.5	8.8	-	-
	500	5	84.5	14.3	-	-
		Mean:	87.2	10.6		
160 pg	50	2	100.9	7.75	99.8	7.0
	100	2	94.5	6.4	99.2	9.9
	200	2	100.0	14.25	93.9	13.4
	500	5	90.3	8.1	-	-
		Mean:	96.4	9.1	97.6	6.8
640 pg	50	2	90.1	8.2	98.1	7.3
	100	2	90.2	5.6	92.1	7.5
	200	2	95.2	8.3	97.5	4.7
	500	5	91.8	2.7	-	-
		Mean:	92.4	6.2	95.9	6.5
Overall mean $\pm$ S.D.			92.2	8.6	96.8	6.6

recoveries obtained with [^{3}H] -progesterone and provide further evidence that the assay is free of distortion of the plasma dose-response curve by solvent residues. The absence of such distortion was further shown by the finding (Table 14) that plasma samples containing high concentrations of endogenous progesterone gave identical concentrations when different volumes covering the entire working range of the dose-response curve were extracted and assayed. For reasons of practicality 2 ml of solvent was used even when volumes below 200μl of plasma were being extracted. The fact that these gave values identical with those found when 200μl of plasma was extracted suggested also that no important interference was produced by increasing the solvent/plasma ratio to above the minimum of 10/1 regularly used.

Specificity of assay for plasma progesterone:

The specificity of the assay for measuring plasma progesterone depends upon a combination of the specificity of the assay end point and the selective nature of the

Table 14 Progesterone Concentration from varied volumes of plasma

Apparent plasma progesterone concentration (ng/ml) was measured in samples from which varied portions were taken and each was extracted with 2 ml of light petroleum. Extraction was performed after the addition of ethanol to plasma (10 vol. plasma + 1 vol. ethanol).

Vol. of plasma (μl)	10	50	100	150	200
Plasma no.	Apparent Progesterone concentration (ng/ml)				
1	14.0	14.8	-	-	-
2	26.0	18.8	-	-	-
3	5.9	-	5.9	-	-
4	17.5	-	17.0	-	-
5	29.0	34.0	-	-	-
6	-	29.4	29.2	28.0	29.7

extraction procedure. The extraction of relevant steroids using the routine procedure is shown in Table 15. In women the plasma levels of 17-hydroxyprogesterone, 20α-hydroxypregn-4en-3-one, 3β-hydroxypregn-5-en-20-one and androst-4-ene-3,17-dione can reach 2.2 ng/ml (Youssefnejadian et al 1972 b), 4 ng/ml (Florensa and Sommerville, 1973), 2 ng/ml (Abraham et al, 1973) and 2 ng/ml (Judd and Yen, 1973), but such values are recorded only during the mid-luteal phase and their equivalents at the assay end point (see Table 9) would amount respectively to 26,40,3 and 0.4 pg of progesterone/ml which would be insignificant at that time. In men and postmenopausal women the maximum concentration of 20α-hydroxypregn-4-en-3-one and 3β-hydroxypregn-5-en-20-one is about one-tenth of those found in the female luteal-phase, so that here again they would not contribute more than 5% of the progesterone as measured. Also in men plasma 17-hydroxyprogesterone concentrations show a mean of 1.23 ng/ml, whereas the highest value reported was 3.2 ng/ml (Youssefnejadian et al 1972 b). These would give responses equivalent to 14.8 pg and 38.4 pg apparent progesterone/ml. As such they could represent a small

Table 15 Extraction of ^{3}H-labelled steroids from male plasma by light petroleum (1 vol. plasma + 10 vol. light petroleum) with and without prior addition of ethanol to plasma

Samples of plasma (500 ul) were equilibrated with dried ^{3}H-labelled steroids for 1 hour and extraction with light petroleum was performed 15 minutes after the addition of ethanol (10 vol. plasma + 1 vol. ethanol). Four replicates were used for each steroid.

	Recovery (%, ± S.D.)	
	Without ethanol	With ethanol
Progesterone	81.5 ± 9.4	92.8 ± 2.0
Cortisol	0.55 ± 0.15	0.29 ± 0.03
3 β-Hydroxypregn-5-en-3-one	84.3 ± 10.8	90.9 ± 1.3
20α-Hydroxypregn-4-en-3-one	78.1 ± 7.0	88.0 ± 4.0
Androst-4-ene-3, 17-dione	77.8 ± 6.5	90.1 ± 1.7
3 β-Hydroxyandrost-5-en-17-one	76.3 ± 7.1	89.1 ± 2.1
17-Hydroxyprogesterone	65.2 ± 7.4	78.9 ± 1.8

intrusion into the assay's specificity, which should, however, be generally acceptable since the mean recorded value in men was 205 pg/ml.

The more polar adrenal steroids are poorly extracted by light petroleum and as judged from the extraction of cortisol (Table 15) are likely to be even less well extracted when ethanol is first added to the plasma.

The specificity with respect to cholesterol was further investigated. Samples from each of six hypercholesterolaemic men and two post-menopausal women whose plasma cholesterol concentrations ranged from 3.7 to 4.6 mg/ml and three with cholesterol concentrations ranging from 0.87 to 1.0 mg/ml gave progesterone values of 72-220 pg/ml, which were not different from those of the normal group. Although this would suggest that the specificity of the progesterone assay was maintained because of poor extraction of cholesterol, direct evidence from its measurement (Abell et al, 1952) in the light petroleum extracts indicated that these contained almost all of the total plasma cholesterol. This suggested that the response obtained in the specificity studies

represented in Table 9 and consisting of a small fall in the percentage of [^{3}H]progesterone bound to antibody when cholesterol was present at 10 µg/ml (which was at the limit of its solubility in the diluent solution) did not represent a true cross reaction.

Table 16 gives a list of certain steroids and shows the effect of the combination of their specificity and blood concentration on the present progesterone assay. These steroids when present in their highest reported concentrations under normal conditions or under stress, contribute a total of 68.5 pg and 138 pg respectively measured as progesterone and, as such, should interfere in the assay especially when low levels of progesterone are being measured. However, six aliquots each from follicular-phase pool plasma (500 µl), luteal-phase pool plasma (50 µl) and a mixture of luteal-phase and male plasma (100 µl) used as quality control for all assays, were extracted by the routine procedure; in each case four were assayed direct and the remainder were subjected to gel filtration on Sephadex LH-20 using the solvent system of Murphy (1971). The eluates having the elution characteristics of added non-radioactive and [^{3}H]progesterone

Table 16 End Point Specificity of the Assay

Steroids with limited specificity or those with relatively high blood concentrations	Maximum Normal Levels and Maximum Reported Under Stress ng/ml	% Cross Reaction with Antibody R 1 (4th booster)	Progesterone Equivalent Un-extracted in pg	% Radioactive steroid extracted by the routine procedure	Progesterone equivalent extracted in pg.
Cortisol	140 ± 5 (S.D.) (West et al 1973) under stress of pulmonary insufficiency 720 ng/ml (Srivastava et al. 1973)	0.02	28 72.0	0.55	0.15 0.44
Pregnenolone	1.38 ± 0.64 (S.D.) Luteal Phase (Abraham et al 1973) Under ACTH stimulation levels in males up to 4.5 times at 15 min (9ng/ml) (Bermudez and Lipsett 1972)	0.13	2.6 11.7	91.00	2.4 11.0
20α-Hydroxypregn-4-en-3-one	4 ng/ml (Luteal phase) (Florensa and Sommerville 1973)	1.0	40.0	88.00	35.2
Androst-4-ene-3,17-dione	1.93 ± 0.06 (S.E.) Mid luteal phase (Judd and Yen 1973)	0.02	0.4	90.00	0.4
Dehydro-epiandrosterone DHA	Follicular phase 9.43 ng/ml Abraham et al 1973	0.004	0.4	89.00	0.36
17-Hydroxyprogesterone	2.2 ng/ml in luteal phase 3.2 ng/ml in men (Youssefnejadian et al 1972a) under HCG levels up 3 folds in men (Strott et al 1969) under ACTH levels up 3 folds in men Bermudez and Lipsett 1972	1.20	26.4 38.4 115.2	79.00	21.0 30.3 91.0
Maxm. Normal Intrusion			110.0		68.5
Maxm. Intrusion under stress			240.0		138.4

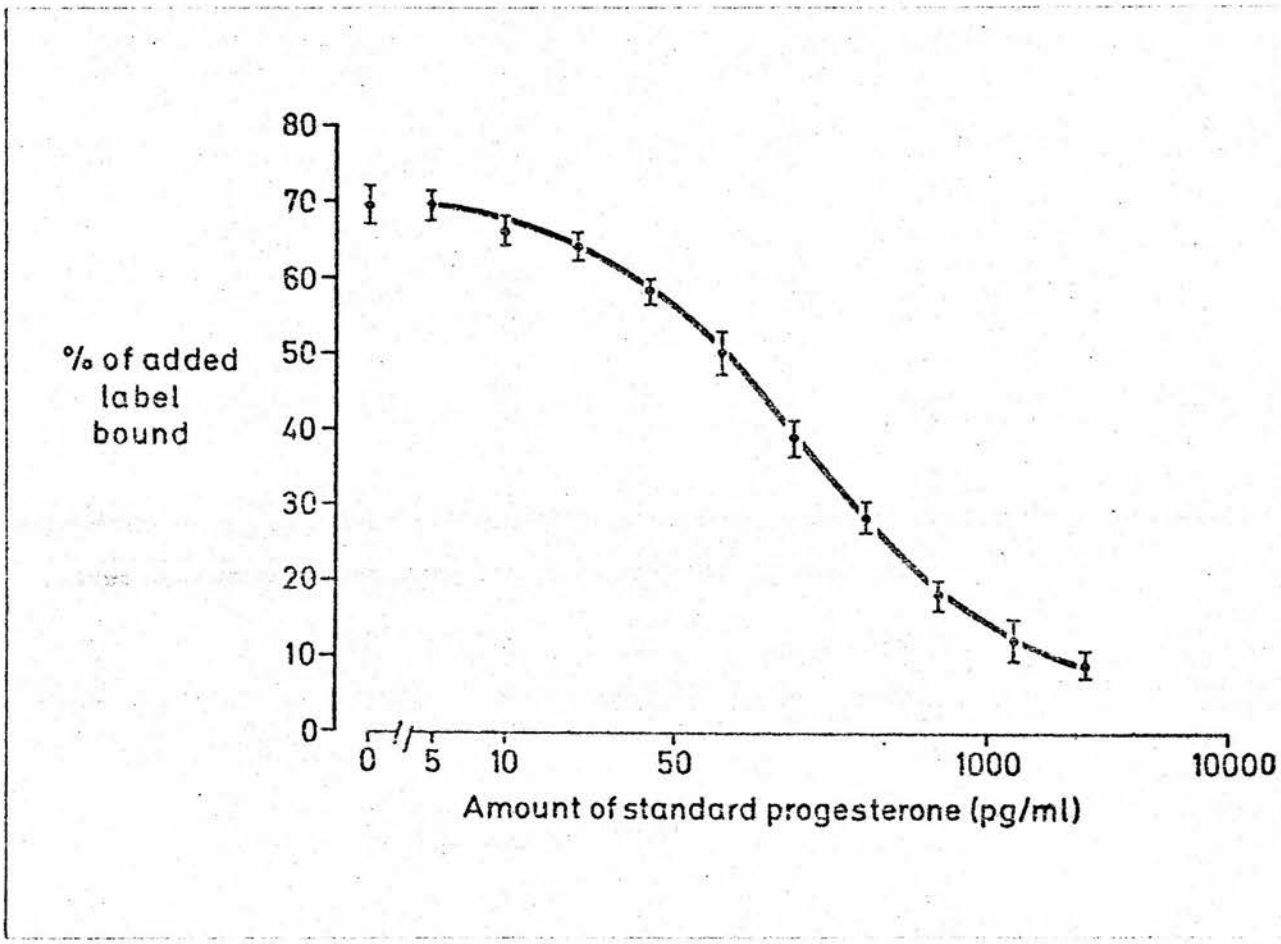
gave mean values of 0.305, 14.0 and 3.0 ng/ml respectively, while those measured directly gave means of 0.297, 14.0 and 2.82 ng/ml respectively. 20∝-Hydroxypregn-4-en-3-one and 17-hydroxyprogesterone, whose cross-reactions with the progesterone antiserum contribute significantly to the non-specificity in the assay (as shown in the Table 16) are clearly separated in the solvent system on the Sephadex-LH 20 chromatography.

Precision of Assay:

The intra-assay precision was assessed by calculating the coefficient of variation from the duplicate determinations on 203 plasma samples, whose values ranged from 85 pg to 35 ng/ml, which were assayed in the ten consecutive assays whose standard curves are represented in fig. 16 . Using the formula, coefficient of variation $= \sqrt{\frac{\Sigma(\frac{d}{\bar{x}} \times 100)^2}{2n}}$ where d = difference between duplicate estimations, $\bar{x}$ = mean of duplicate estimations and n = number of duplicate estimations, a value of 9.7% was found. The inter-assay precision was

Fig. 16

Radioimmunoassay standard curve for progesterone from ten consecutive assays



Mean (± S.D.) obtained in 1 ml of final incubation volume from ten consecutive routine assays.

examined by calculating the coefficient of variation of the means of triplicate determinations carried out on a quality control pool in the ten consecutive assays. A mean Value of 2.78 ng/ml was found and the coefficient of variation was 5.0 %. In five of these assays the same pool was assayed a second time (again in triplicate), the two sets of tubes being placed at the beginning and end of the assays. Mean values of 2.76 and 2.98 ng/ml respectively were obtained.

Sensitivity and Working Range of the Routine Assay:

Sensitivity has been defined as that amount of substance being assayed which gives a fall in percentage binding of tracer of 2.5 x S.D. of percentage of tracer bound in the absence of unlabelled progesterone (Borth 1970). Fig. 16 shows the mean standard curve (± S.D.) calculated from ten consecutive routine assays. The sensitivity of the assay by the above formula is 11 pg/tube for the routine 1 ml incubation mixture. However, the slope of the standard curve is still shallow at the above formal detection limit of 11 pg/ml and precision is therefore less than optimal. It has been preferred therefore to discard values derived from responses which

were 10% below the 0 standards that is, the binding of tracer in the absence of unlabelled progesterone. When considered in this way the working range for the routine assay (with 1 ml incubation volumes) is 33 - 600 pg. This provides for a range of 165 - 3000 pg/ml of plasma when 0.2 ml is extracted.

Assay of progesterone-like activity in urine:

Introduction:

Attempts to measure progesterone in urine began more than 40 years ago when the occurrence of biologically active "progesterone-like" material in urine was reported (De Fremery et al. 1931). Its identification as progesterone using physio-chemical techniques, however, was not proved even when extracts of 10,000 gallons of human pregnancy urine was employed for this purpose (Marker 1937). Progesterone remained unidentified in urine even when more sensitive techniques like gas liquid chromatography (GLC) were used for its measurement (Drodowsky et al. 1965). After injection of radioactive progesterone in a non-pregnant subject these workers

isolated 0.01% of the radioactivity in the urinary progesterone fraction. Ismail and Harkness (1967) isolated progesterone for the first time in a pooled human pregnancy urine. The identity of their isolated compound as progesterone was based on its: behaviour in different chromatographic systems; formation of specific chemical products and the infra red spectrum. Later, the isolation and identification of progesterone in the urine of non-pregnant human subjects was reported by Van der Molen and Corpechot (1968). They identified the isolated compound as progesterone on the basis of: its specificity of behaviour in 3 different column chromatography systems and several paper chromatography systems; its resistance to acetylation; the ability to reduce to 20 β-hydroxy steroid by the appropriate enzyme; the behaviour of this reduced chloroacetylated compound in T.L.C. and GLC systems; and finally its crystallization to constant specific radio activity with 20 β-hydroxyprogesterone-chloroacetate of the radioactive compound which was isolated from the urine of subjects after the intra-venous injection of radioactive progesterone. On the basis of the injected radioactivity they calculated a 24 h excretion rate of 0.05 - 0.5 μg in the urine of non-pregnant women.

With the availability of the present sensitive RIA method for measuring progesterone in plasma it was of interest to apply this method for the assay of progesterone in urine of the normally menstruating women.

Dried petroleum ether extracts in duplicate from follicular and luteal-phase urinary pools (0.5 ml aliquots) were taken with another duplicate set of follicular-phase aliquots containing unlabelled progesterone. These were further subjected to Sephadex LH-20 chromatography purification and each fraction from these columns was assayed by the standard radioimmunoassay procedure. Radioactive progesterone was also added to the follicular phase urine pool and the dried petroleum ether extract containing the radioactivity was similarly purified on Sephadex-LH-20 column. Fig. 17 shows the behaviour of the progesterone-like activity in follicular and luteal phase urinary extracts on the Sephadex LH-20 column. As shown in the fig. 17 this progesterone-like activity in the two phases in urine behaves identically on the Sephadex LH-20 column to that of the added unlabelled and the radioactive progesterone.

Fig. 17

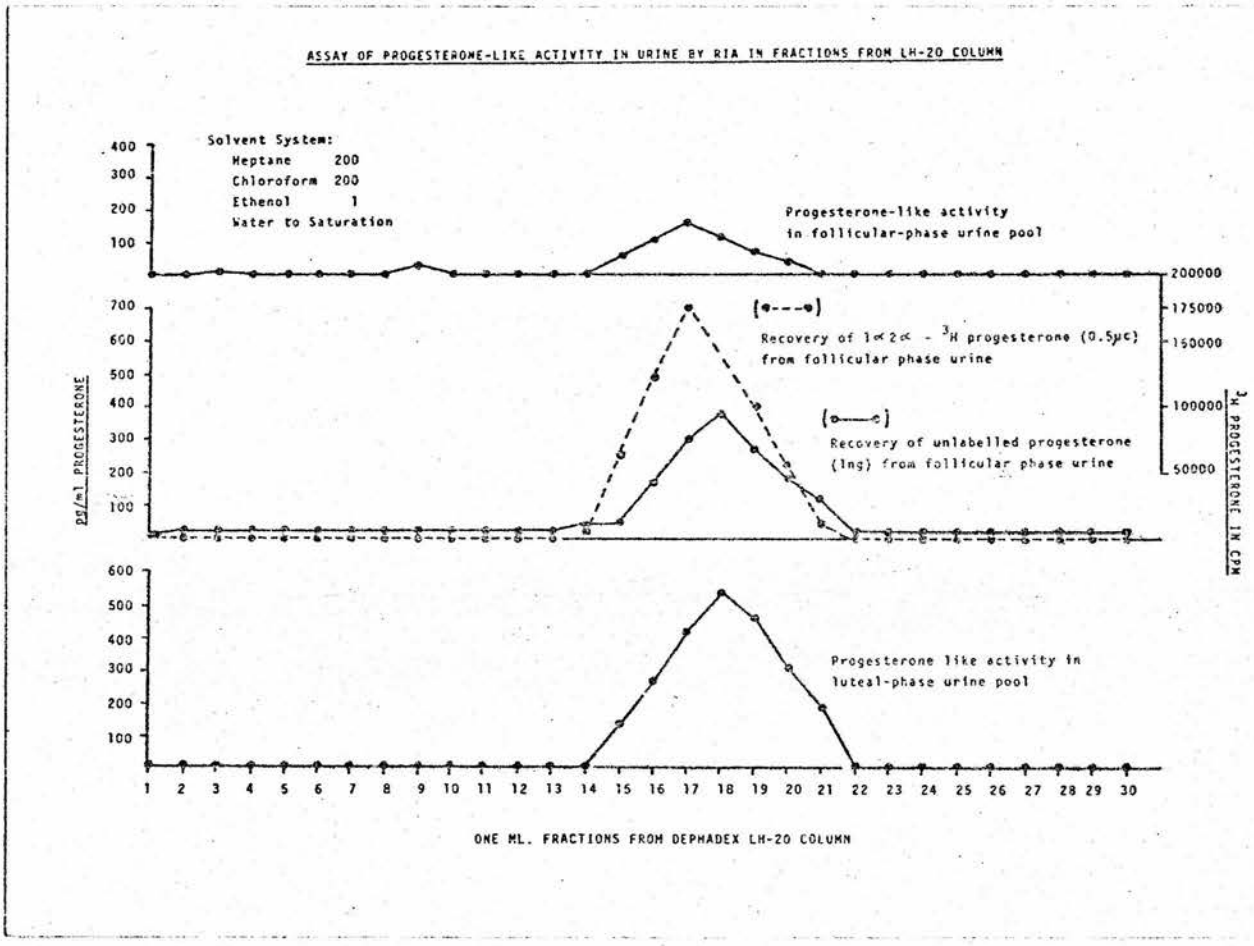
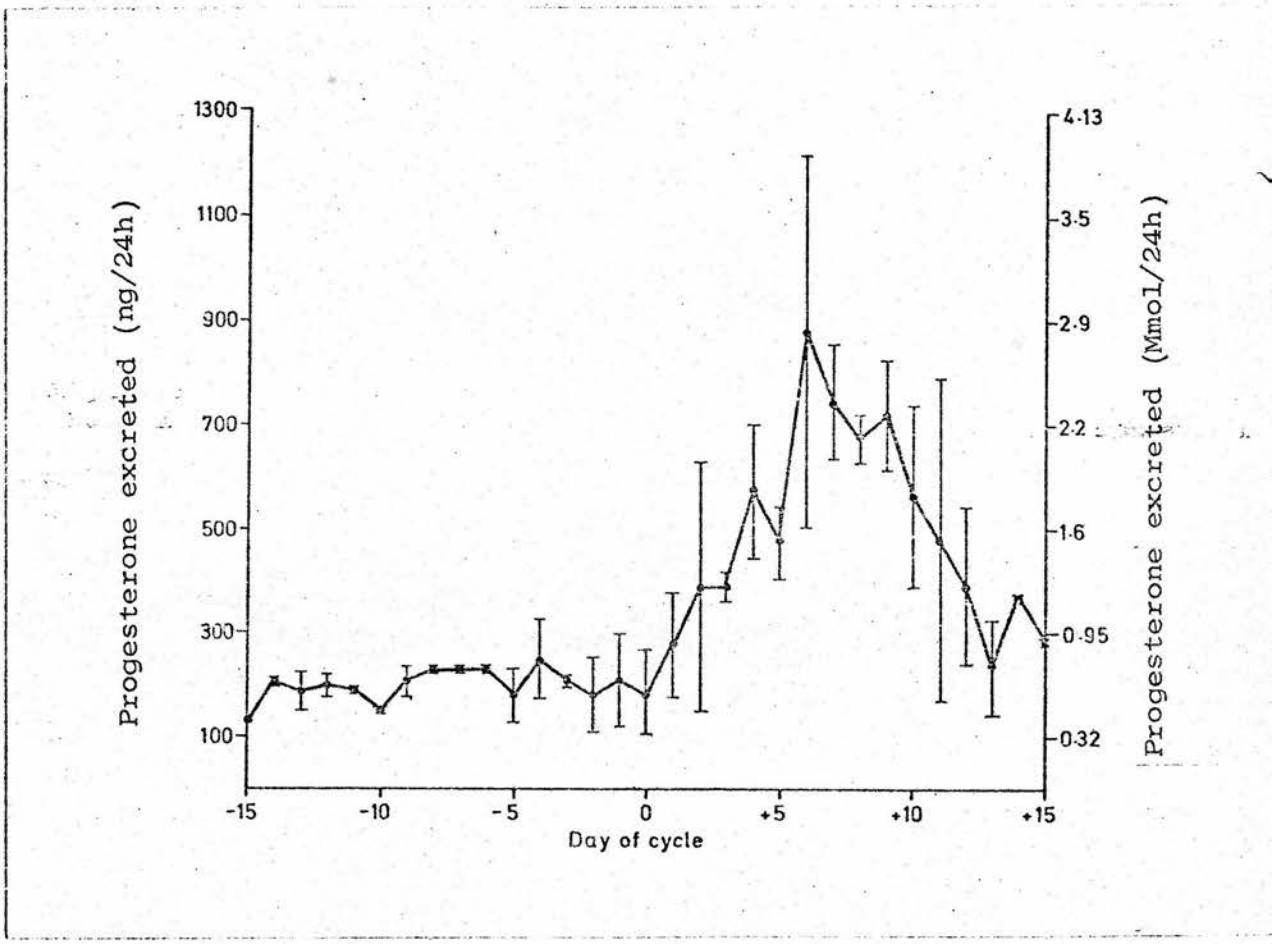

ASSAY OF PROGESTERONE-LIKE ACTIVITY IN URINE BY RIA IN FRACTIONS FROM LH-20 COLUMN
Solvent System:
Heptane 200
Chloroform 200
Ethenol 1
Water to Saturation
Progesterone-like activity in follicular-phase urine pool
Recovery of 1∝2∝ - ³H progesterone (0.5μc) from follicular phase urine
Recovery of unlabelled progesterone (1ng) from follicular phase urine
Progesterone like activity in luteal-phase urine pool
pg/ml PROGESTERONE
³H PROGESTERONE IN CPM
ONE ML. FRACTIONS FROM DEPHADEX LH-20 COLUMN

Fig. 18 shows the values obtained in 3 women from 24 h urinary samples collected daily throughout the complete menstrual cycles. The ratios between luteal-phase and follicular-phase excretion rates (4:1) are smaller than the comparable ratios from plasma from the same subjects (20:1). The urinary excretion rates of 4:1 were maintained when these assays were repeated using the 6 β-hydroxyprogesterone-BSA antiserum (R 390 5th bleed).

This discrepancy between follicular and luteal-phase ratios in plasma and urine could probably be explained on the basis of two factors: (1) that of the non-specificity of the method in urine resulting in contributions to the urinary progesterone values by substances other than progesterone providing a base-line showing false high values which would be especially noticeable in the follicular phase, or (2) that the urinary excretion rate was disproportionately higher in the follicular-phase. That the second explanation was perhaps the more probable one was suggested by the finding that the progesterone-like activity from pools of follicular and luteal-phase urine samples which were run on Sephadex-LH-20 columns (Murphy 1971) gave values of 0.475 and 1.79 ng/ml which were identical with values

Fig. 18



Mean urinary excretion (± S.D) of progesterone-like activity in 24h samples from three menstrual cycles

Day 0 is the centre of the mid-cycle urinary luteinizing-hormone surge.

of 0.479 and 1.78 ng/ml obtained following simple petroleum ether extraction.

Behaviour of Unextracted Plasma in the Assay:

In the course of this work it became apparent that the specificities of progesterone antisera were such as to be theoretically usable for assaying progesterone in unextracted plasma. If all the compounds listed in Table 9 were present together in plasma at their maximum concentrations and exerted additive effects on the specificity of the method then the progesterone equivalent of their combined effect will be in the region of 250 - 300 pg/ml (see also Table 16). Such a high level of non-specificity would be unacceptable in situations of low progesterone concentration as found in follicular phase or in men, but it may be quite acceptable in situations of high progesterone secretion such as, for monitoring the presence and function of the corpus luteum.

Assays were performed simultaneously on extracted and unextracted plasmas using 11α-hydroxyprogesterone-BSA and

6 β-hydroxyprogesterone-BSA antisera. Plasma pools so assayed from (a) normal men (b) postmenopausal women (c) luteal-phase and (d) luteal-phase plasma diluted with male plasma as quality control gave grossly high values in unextracted plasma as shown in Table 17 and 18 . Each pool of plasma was measured in differing aliquots and the dose response curves for different quantities of each pool were parallel to those of the progesterone standards. The high values in the unextracted plasma remained essentially unaltered when plasma was diluted or when the diluted plasma was heated for 60 min at 100°C, centrifuged and the supernatant assayed. The latter procedure was adopted to inactivate the plasma binding proteins and to free the plasma from lipids. As the values in unextracted plasmas have remained essentially unaltered by this treatment two possible sources of the non specificity are suggested. One is that the binding proteins were not inactivated and were competing with the labelled progesterone (giving false low %B); alternatively the high values might have been due to other unidentified steroids or metabolites whose cross reactions with these antisera were not tested.

Table 17 Assay of Progesterone in Un-Extracted and Extracted Plasma Pools by Progesterone -11α-BSA Antiserum

Plasma Source	PROGESTERONE in Unextracted Plasma ng/ml					Extracted ng/ml mean
	Plasma Aliquots				Mean	
	20µl	50µl	100µl	200µl		
Male Plasma Pool						
1 Neat Plasma n = 4	-	2.0	1.95	1.85	1.93	
2 Plasma Diluted x 4 times	-	-	1.0	1.74	1.4	0.12
3 Plasma heated at 100°C centrifuged & supernatent assayed n = 4	-	-	1.28	1.84	1.6	
Post Menopausal - Plasma Pool						
1 Neat Plasma n = 4	-	3.8	3.9	3.95	3.9	
2 Plasma Diluted x 4 times	-	-	2.2	2.7	2.45	0.09
3 Plasma heated at 100°C centrifuged & supernatent assayed n = 4	-	-	2.1	2.9	2.5	
Luteal Phase Plasma Pool						
1 Neat Plasma n = 4	13.75	14.30	13.70	Beyond range of the std. curve	13.9	
2 Plasma diluted x 4 times	-	28.8	31.6	19.2	26.5	17.2
3 Plasma heated at 100°C centrifuged & supernatent assayed n = 4	-	9.6	7.6	7.5	8.2	
Quality Control Plasma Pool						
1 Neat Plasma n = 4	10.25	9.5	9.8	Beyond range of the std. curve	9.85	
2 Plasma diluted x 4 times	-	7.4	4.4	4.0	5.3	2.9
3 Plasma heated at 100°C centrifuged & supernatent assayed n = 4	-	7.0	3.7	3.7	4.8	

Table 18 Assay of Progesterone in Un-Extracted and Extracted Plasma Pools Using Progesterone-6 β BSA Antiserum

	PROGESTERONE ng/ml in Unextracted Plasma					Extracted
	Plasma		Aliquots		Mean	
Male Plasma Pool	20µl	50µl	100µl	200µl		
1 Neat plasma n=4	-	3.65	3.3	3.25	3.4	
2 Plasma diluted x 4 times	-	-	2.6	2.88	2.74	0.1
3 Plasma diluted x 4 times, heated at 100°C centrifuged & supernatent assayed	-	-	6.4	5.6	6.0	
Post Menopausal Plasma Pool						
1 Neat plasma n = 4	-	4.15	4.10	4.75	4.3	
2 Plasma diluted x 4 times	-	-	4.7	4.5	4.6	0.08
3 Plasma diluted x 4 times, heated at 100°C centrifuged & supernatent assayed	-	-	5.4	4.3	4.85	
Luteal Phase Plasma Pool						
1 Neat plasma n = 4	26.5	23.4	High Beyond range of std. curve		24.95	13.2
2 Plasma diluted x 4 times	-	40.4	30.0	High	35.2	
3 Plasma diluted x 4 times, heated at 100°C centrifuged & supernatent assayed	-	44.8	46.0	30.0	40.3	
Quality control plasma						
1 Neat plasma n = 4	10.0	8.6	8.6	High	9.1	
2 Plasma diluted x 4 times	-	9.2	6.6	4.9	6.9	2.3
3 Plasma diluted x 4 times, heated at 100°C centrifuged & supernatent assayed	-	14.0	11.6	8.8	11.5	

SECTION 3

Results and Discussion

6. Applications.

Applications of the plasma progesterone assay method:

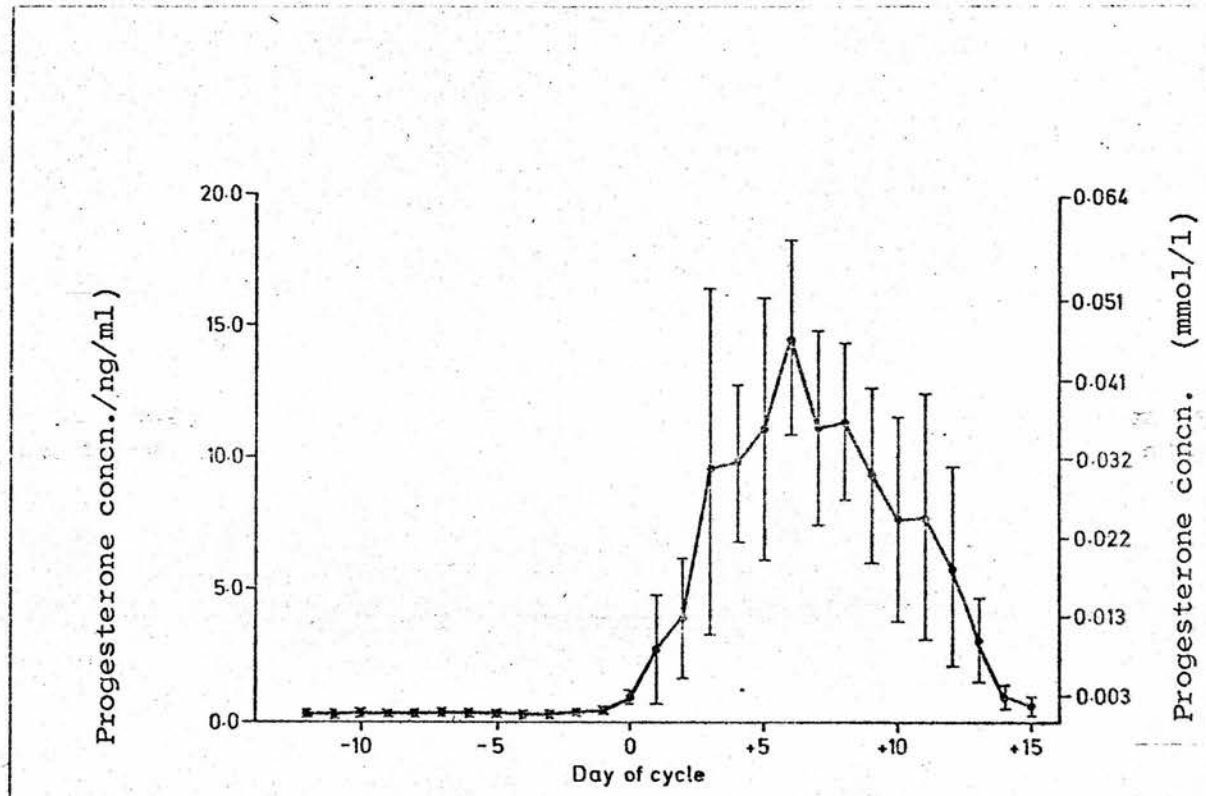
The assay method has been applied to measure plasma progesterone levels in certain steady state situations and in some physiological studies.

Duplicate 0.5 ml aliquots of single samples from each of 50 normal men were estimated and the progesterone value found was 205 ± 20.6 (SD) pg/ml. The value found for a pool of plasma from 3 post menopausal women was 88 pg/ml.

1 Progesterone levels in the normal menstrual cycle:

Fig. 19 shows the mean (± S.D.) of plasma progesterone levels found from daily sampling throughout a single menstrual cycle from 5 normal women, day 0 being the centre of the midcycle plasma LH surge. The mean base line levels in the follicular-phase was 332 pg/ml and a mean luteal-phase peak of 14.6 ± 3.7 (S.D.) ng/ml was found on the 6th day after the LH peak. The progesterone values obtained in the menstrual cycle and the values in men and post menopausal women have been compared with the previously published values by other radioimmuno assay methods and shown in table 19 .

Fig. 19



Mean plasma progesterone concentration (± S.D.) in five menstrual cycles.

Day O is the centre of the mid-cycle plasma luteinizing-hormone surge.

As shown in the table[19] progesterone levels in the follicular-phase as reported by Abraham et al 1971, De Villa et al 1972, and Kutas et al 1972, are higher than those reported by others. Abraham et al (1971) used a antiserum raised to the 21-position of the 11-deoxycortisol with a cross-reaction of 100% with progesterone. In this method progesterone is separated from other cross-reacting steroids, except cholesterol with which the antiserum cross reaction is reported at less than 0.01%, by chromatography on a Celite column. The high values (545 ± 103 pg/ml) as reported by these workers may be due to the nonspecific intrusion from steroids not tested in the system or from cholesterol whose plasma concentration is nearly 10,000,000 times (2 mg/ml) that of progesterone. The follicular-phase levels of progesterone at 550 ± 440 pg/ml (S.D.) for days 2 - 10 as reported by De Villa et al (1972) are difficult to explain since the specificity of their assay end-point was well tested and also the precision of their measurement was good (with a inter and intra-assay precision of 8%). They however did not test the cross-reaction of their antiserum with cholesterol and it is also likely that the majority of their samples (16 determinations) were from nearer day 10 when more mature

Table 19 Plasma Progesterone Values as Reported by Various Authors

		Abraham et al. (1971)	Furuyama & Nugent (1971)	Thorneycroft & Stone (1972)	De Villa et al (1972)	Kutas et al. (1972) (plasma pools)	Youssef-nejadian et al. (1972)	Cameron and Scaris-brick (1973)	Prese Metho
Whether chromatography employed		Yes	Yes	No	No	No	Yes	No	No
Men	pg/ml	233 ± 64	266 ± 97 (S.D.)	193	260 ± 80 (S.D.)	195	230 ± 6.8 (S.D.)	-	205±20. (S.D.)
Post-menopausal women	pg/ml	180 ± 63 (S.D.)	-	63 - 225	260 ± 80 (S.D.)	-	-	-	88.0±8.
Women follicular phase	pg/ml	545 ± 103 (S.D.)	265 ± 192 (S.D.)	60 - 371	500 ± 440 (S.D.)	933 ± 86 (S.D.)	424 ± 187 (S.D.)	120 ± 70*	331.7 187.5 (S.D.)
Women luteal phase	ng/ml	8.56 ± 4.7 (S.D.)	8.3 ± 6.3 (S.D.)	8.9 (4.3 - 19.4)	12.1 ± 4.81	5.6 ± 2.1	7.7 ± 1.9	15.8 ± 6.7Δ	7.32±4. (S.D.)

* 7 days before ovulation

Δ 7 days after ovulation

follicles are present. Taking into account the good sensitivity and specificity of the method the values of follicular phase progesterone at 933 ± 86 (S.D.) as reported by Kutas et al (1972) represents more probably the periovulatory state of the follicle.

The mean luteal-phase values of 7.32 ± 4.26 (S.D.) ng/ml as reported by the present method are comparable with those of other methods.

As shown in fig. 19 the first significant rise in plasma progesterone levels occurs on the day of the LH peak and this is in agreement with majority of published studies where the first rise in progesterone was observed after the start of the LH surge (Johansson and Wide 1969, Vandewiele et al., 1970, Abraham et al. 1972, Dupon et al. 1973). This observation is consistent with the presently held view that the mid-cycle peak of LH is not initiated by progesterone.

Physiological Studies:

Progesterone is an intermediate in the biosynthesis of adreno-cortical and gonadal hormones. However the evidence so far suggests that it plays a minor role in the biosynthesis of cortisol in the adrenals. Its function in females as one of the major secretory products of the ovary is well established but the evidence so far indicates that it plays only a minor role in the synthesis of the main testicular androgens in the male (see Chapter 1). The effect of stimulation and supression of the adrenals on the circulating progesterone levels in males was studied in collaboration with Dr. A. Toft of the Royal Infirmary, Edinburgh. The study of the secretory pattern of progesterone in males was studied in collaboration with the MRC Unit of Reproductive Biology.

1. Effect of ACTH Stimulation on Progesterone Levels in Males:

Two normal male volunteers were given an intramuscular dose of 1 mg of 1-18 -ACTH and samples were taken every 15 min. from two hours before to four hours after the ACTH administration.

Plasma progesterone was measured in duplicate and Table 20 gives their mean values for the ACTH stimulation experiments. After ACTH administration progesterone levels showed the following pattern: a two-fold rise in one subject (W) was observed after one hour, the rise in levels going up to two and a half fold in 1.75 hours then three-fold after 2.75 hours and remaining at these levels up to four hours after the ACTH injection. In the other subject (T) the progesterone concentration at one hour after ACTH injection was up two-fold and after 1.5 hours three-fold higher than basal levels. The progesterone levels were four-fold at 3.0 hours then rose to six-fold at 3.5 hours and remained at these levels at four hours after the ACTH injection when the blood sampling was stopped.

The results show that the progesterone production is increased as a result of ACTH stimulation and suggest the ability of the adrenal cortex to synthesize progesterone in response to ACTH stimulation. A four-fold rise in progesterone concentration in response to continuous infusion of ACTH has been previously reported (Strott et al. 1969). In the present studies the progesterone

Table 20 Effect of ACTH Stimulation on Progesterone Levels in Males

Time Before and After ACTH Administration in Hours	Plasma Progesterone Levels (pg/ml) Subjects W	T
- 2 hours	199.0	270.0
- 1·75	240.0	272.0
- 1·50	188.0	233.0
- 1·25	175.0	220.0
- 1 hour	220.0	268.0
- 0·75	150.0	225.0
- 0·50	182.0	220.0
- 0·25	188.0	223.0
0 hour	208.0	225.0
+ 0·25	232.0	315.0
+ 0·50	256.0	525.0
+ 0·75	340.0	390.0
+ 1.00 hour	395.0	440.0
+ 1·25	400.0	590.0
+ 1·50	480.0	615.0
+ 1·75	490.0	520.0
+ 2.00 hours	490.0	580.0
+ 2·25	490.0	385.0
+ 2·50	440.0	490.0
+ 2·75	700.0	640.0
+ 3.00 hours	555.0	860.0
+ 3·25	620.0	730.0
+ 3·50	570.0	1205.0
+ 3·75	615.0	1030.0
+ 4.00 hours	640.0	1255.0

levels were still rising up to four hours after being stimulated with ACTH which has a short-life of 5-25 min(Besser et al 1971 The slow rise in the progesterone levels may be due to the intramuscularly administered slow acting synthetic preparation of ACTH used or that it may suggest a slow rate of synthesis of progesterone. This is also suggested by the studies of Bermudez and Lipsett (1972) who administered ACTH to five normal men by continuous infusion and sampled the blood at frequent intervals. It was shown that the levels of steroids of the $\triangle^5$-pathway, pregnenolone and 17-hydroxy pregnenolone increased at a faster rate showing larger responses than that of progesterone measured simultaneously indicating that progesterone was not the important intermediate on pathway in the synthesis of cortisol. The present studies thus support the results of the above workers insofar as the slow synthesis of progesterone is concerned.

2. Effect of dexamethasone Suppression on the Progesterone Levels in Men:

Two normal adult male volunteers were given 2 mg dexamethasone every six hours starting at 11 p.m. Blood

samples were collected over a six hours period ten hours after the first dose of dexamethasone and a single sample was taken after 24 hours. The means of duplicate determinations of progesterone levels on these samples are shown in Table 21 . For this experiment the appropriate controls and plasma samples taken before the start of the dexamethasone test were not available so the extent of reduction in the circulating levels of progesterone following dexamethasone is not known. The results in Table 21 show that significant concentrations of progesterone are present following dexamethasone suppression. The progesterone responses in the assay correspond to 12 - 15% inhibition of tracer binding and therefore are clearly above the sensitivity limit of the assay (10% inhibition of the tracer binding).

It has been suggested that progesterone levels in males are mainly adrenal in origin as the production rates in males and ovariectomised women are not significantly different (Little et al., 1966). It has been shown that the high levels of progesterone in children with congenital adrenal hyperplasia can be suppressed by glucocorticoids (Strott et al., 1970)

Table 21 Plasma Progesterone levels during Dexamethasone suppression in Males

Time in Hours	Plasma Progesterone Levels (pg) Subjects	
	W	C
0.00 hour	167.0	140.0
+ 0·25	147.0	-
+ 0·50	138.0	-
+ 1.00	182.0	108.0
+ 2.00	119.0	90.0
+ 3.00	90.0	97.0
+ 4.00	90.0	131.0
+ 5.00	117.0	117.0
+ 6.00	138.0	60.0
+ 24.00	209.0	90.0

Dose regimen: 2 mg dexamethasone at 2300 and 0600 hours

1 mg dexamethasone at 1200 hours and 6 hourly thereafter

suggesting that progesterone levels of adrenal origin are under the control of ACTH. The measurable progesterone response in the present studies after the suppression of ACTH by dexamethasone can be explained as follows: (a) As progesterone is not an important intermediate in the synthesis of cortisol, its levels (as shown in Table 21) may reflect residual adrenal secretion which is not suppressed by dexamethasone. (b) There may be a testicular contribution to the circulating levels of progesterone, but this is not likely to be significant as progesterone concentrations have been shown to remain unaffected in response to stimulation with HCG or after suppression of the testis with fluoxymesterone (Strott et al. 1969). (c) Lastly, unless a portion of the circulating levels of progesterone is under the control of other pituitary hormones in addition to ACTH, the progesterone response in the assay may represent a non-specific intrusion by cross reacting steroids. This seems more likely because the responses as obtained are limited in the upper part of the dose-response curve where cross-reactions with steroids such as cortisol, or 17 hydroxyprogesterone which is secreted by the testis,

because of their non-parallel curves, are more intrusive than at the lower part of the response curve.

3. The Mode of Secretion of Progesterone in Males:

Recent evidence has suggested that some hormones are secreted episodically rather than continuously. Cortisol was the first steroid hormone shown to secrete episodically (Weitzman et al., 1966). The secretion of luteinising hormone (LH) has also been shown to be pulsatile in nature (Santen and Bardin 1973). It was of interest to study the pattern of secretion of progesterone and compare this to the pattern of secretion of testosterone, LH and follicular-stimulating hormone (FSH) to investigate their relationships in the study of pituitary-gonadal axis in males.

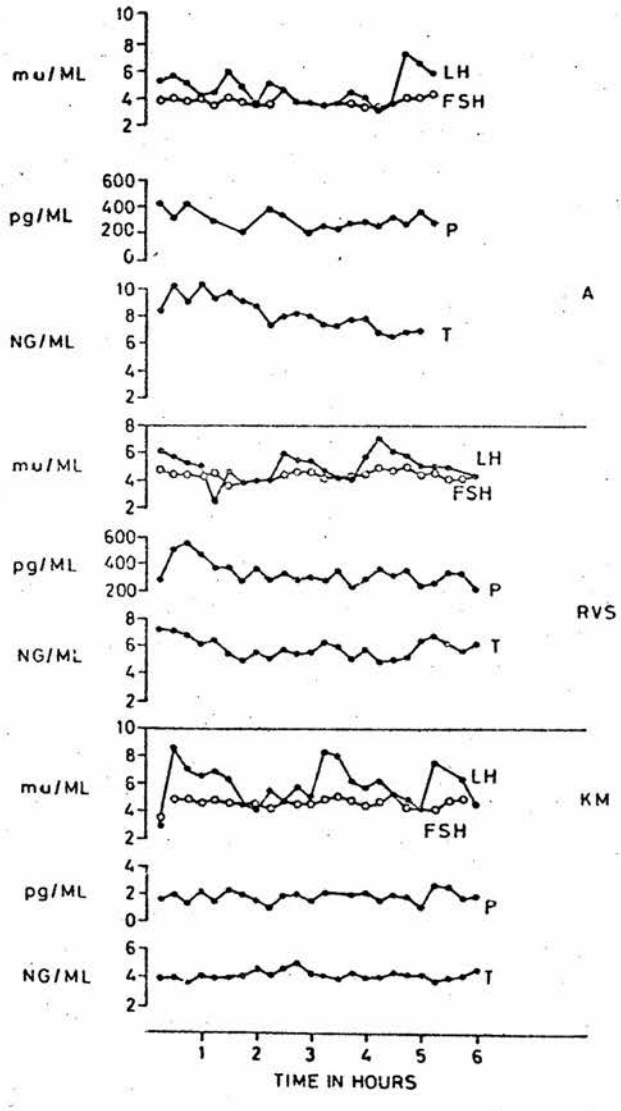
Progesterone levels were measured in blood samples collected every 15 minutes over a six hour period from three normal males. The plasma levels of LH and FSH were measured by Dr. W.M. Hunter of the M.R.C. Radio Immuno Assay Team and plasma testosterone levels were by Dr. C. Corker of the M.R.C. Unit of Reproductive Biology.

The pattern of secretion of LH, FSH, testosterone and progesterone is shown in fig. 20 . The within assay precision of duplicate measurements of progesterone calculated as the coefficient of variation was 14.3% (KM), 11.9% (RVS) and 10.0% (A) and the coefficient of variation between duplicate determinations over the six hour period in each subject was 25.3%, 26.8% and 25.9% respectively suggesting that progesterone is not secreted at a steady rate. However these results do not suggest a regular pattern due to episodic secretion of progesterone.

The episodic manner of secretion of progesterone could conceivably be involved in the gonadal or adrenal steroid feedback control of pituitary hormones - principally LH or ACTH. The present studies shown in fig. 20 do not reveal any clear relationship in the pattern of secretion between progesterone, testosterone, LH or FSH. It has been proposed that a hormone is adrenal in origin and under the control of ACTH if it is secreted episodically and in synchrony with cortisol (Katz et al., 1972). Cortisol levels on these samples were not measured however and they therefore provide no information as to whether the variations in the secretory pattern of progesterone are due to its adrenal component.

Fig. 20

STUDY OF SECRETORY PATTERN OF PROGESTERONE IN MALES IN RELATION TO CERTAIN OTHER HORMONES OVER A SIX HOUR PERIOD



P → PROGESTERONE

T → TESTOSTERONE

GENERAL DISCUSSION OF THE METHOD

GENERAL DISCUSSION

The cross-reaction studies with the three antisera to 11∝-hydroxyprogesterone-BSA (Table 9) show that the degree of cross-reactivity found in sera from all of the rabbits are similar to that first shown by Midgley and Niswender (1970) and set out later in greater detail by Niswender (1973). These studies thus confirm the specificities that can be expected of antisera raised to 11∝-hydroxyprogesterone-BSA conjugates. Generally, the specificities of the antisera described in the present work, with respect to 3 β-hydroxypregn-5-en-20-one; 20 β-hydroxypregn-4-ene-3-one, 11-deoxycorticosterone and cortisol are better than most reported. For 20∝-hydroxypregn-4-en-3-one two antisera (Kutas et al., 1972, Niswender 1973) at 0.1% and 0.2% cross reactions are better than the present 0.6 - 2.0%. For 5 β-pregnane-3,20-dione cross-reactions of 5 - 7% with most antisera and 17% with one (R3 4th booster) antiserum compares favourably with reported values of 6% (De Villa et al., 1972), 11% (Thorneycroft and Stone 1972) and 4.8% (Kutas et al., 1972). For 5∝-pregnane-3,20-dione cross-reactions are very varied in the present studies, ranging from 7.5% (R3 4th booster) to 23% (R1 7th and 8th booster) and those reported range from 3.0% (Lindner et

al., 1972), 5.4% (Spieler et al., 1972), 6.5% (Kutas et al., 1972), 13% (Niswender 1973), 15% (De Villa et al., 1972) to 80% (Youssefnejadian et al., 1972).

Detailed specificity studies on antisera raised to 6 β-hydroxyprogesterone-BSA conjugates were also performed (Table 9). These showed less ability to recognize the loss of the double bond at C-4 position than was the case with the 11α-hydroxyprogesterone antisera. For 5 β-pregnane-3,20-dione the cross-reactions were 18% (R 388), 14% (R 389) and 20% (R 390) as compared with the published figure of 8% (Jones and Mason 1974) and 100% (Lindner et al., 1972). For 5α-pregnane-3,20-dione the cross-reactions were 68% (R 388), 55% (R 389) and 57% (R 390) compared to reported values of 52% (Riley et al., 1972), 100% (Lindner et al., 1972), 30% (Niswender 1973) and 67% (Jones and Mason 1974). As expected, the changes close to the site of conjugation (at the 6 β-position) were not recognized by these antisera. Cross reactions for 3 β-hydroxy-pregn-5-en-20-one by these antisera were 8% (R 388), 1% (R 389) and 5% (R 390) which are comparable with figures of 8 - 14% (Lindner et al., 1972),

3% (Niswender 1973), 14% (Jones and Mason 1974) and better than the reported figure of 68% (Riley et al., 1972). Since the plasma levels of pregnenolone are similar in the follicular and luteal-phase (Abraham et al., 1973), the limited specificity of 6 β-hydroxyprogesterone antisera with respect to pregnenolone should still be adequate for measuring progesterone levels to monitor the luteal function. However, since they brought no compensatory improvement in specificity with respect to other closely related compounds which differ from progesterone elsewhere in its structure, the 6 β-hydroxyprogesterone-bovine serum albumin antisera were discarded in favour of the 11α hydroxyprogesterone-bovine serum albumin antisera for the routine assay.

The chances of obtaining satisfactory antisera using the present procedure of immunization may be judged from the following findings. The cross-reactivity of eight key compounds was tested on each of six, four and two different samples respectively from each of three rabbits receiving 11α-hydroxyprogesterone-bovine serum albumin conjugate over periods of 27, 11 and 7 months, and

no significant alterations in specificity occurred. Six of the total of 12 rabbits produced antisera which permitted the establishment of assays of good sensitivity. They did so within 3 - 6 months and once this quality was achieved it was not later lost by any animal. Nine of the 12 rabbits gave antisera which could be used at final dilutions of 1/20000 or more when their sensitivity plateaus were first reached; eight of these animals later gave antisera which were used at 1/40000 - 1/60000, and again once reached such titres never subsequently decreased to any significant extent. The titres obtained by other workers have frequently been one order below this range (Bodley et al., 1973; Spieler et al., 1972; Kutas et al., 1972; Youssefnejadian et al., 1972) and the improvement that has been obtained in the present procedure may have been due to the widely placed time intervals between the immunisation injections.

The two separation procedures used, the previously optimised double-antibody system (Hunter and Ganguli 1971)and the independently developed solid-phase antibody system, do not disrupt the equilibrium established between the primary

reaction with antibody and therefore the necessity for investigating and optimizing a system for this particular application to progesterone was obviated. The use of solid-phase antiserum covalently linked to a solid particular support medium (Wide 1969) seems ideal for running routine assays of progesterone. The reagents required are inexpensive, the activation and coupling procedures are simple and no special optimization is required for individual antisera, the losses in the effective titres of antibody are small and there was no loss of sensitivity when this system was used in place of the double-antibody procedure (Bolton and Hunter 1973). When stored at 4°C batches of covalently coupled antisera were used for periods of up to 5 months without any apparent change in properties. Activated cellulose was also very stable if kept in ampoules under nitrogen. The use of covalently coupled antiserum requires only one centrifugation after the primary incubation giving a high degree of precision suggesting that the "misclassification error" may be small (Rodbard *et al.* 1969). The single disadvantage of having to use a shaker for continuous agitation of the incubation mixtures can be obviated by the addition

of sucrose or dextran to the incubation diluent as the covalently coupled antiserum then remains in suspension because of the increased density and viscosity of the medium (Bolton et al., 1974).

The addition of ethanol to plasma before extracting with light petroleum, as first suggested by Holmdahl and Johansson (1972) for their competitive protein-binding method, has yielded a sufficiently high and constant extraction rate in a single extraction. This has removed the necessity for using two extractions or the use of [^{3}H] progesterone tracer to all individual plasma samples in order to monitor and allow corrections for the variable recovery rates obtained. The later procedure has been used in the majority of published methods. It was noticed that there were two ways in which the extracts of plasma (or of water) could interfere in the assay and this was investigated in detail since there is little detailed evidence given in the literature about such intrusion. A simple purification procedure for use on unselected batches of light petroleum has been devised so that the dependence on a closely defined source of light

petroleum (e.g. see Johansson, 1969) has been made unnecessary.

Of the previous reported assays based on the more specific antibodies to 11α-hydroxyprogesterone those of Thorneycroft and Stone (1972), Kutas et al (1972) and Cameron and Scarisbrick (1973) had sensitivities similar to those found with the present antisera. The concentrations and volumes of reagents used in the routine procedure seems ideal for use in assaying progesterone in plasma from women of reproductive age. Thus, a 200µl portion of plasma permits accurate measurement in the follicular-phase and therefore provides for the detection of the midcycle rise at its inception.

The theoretical specificity of the antisera to the known steroids was such as to prompt investigation into the possibility of assaying progesterone in unextracted plasma. When assays were attempted on unextracted plasma the values obtained were spuriously high by about 2-5 ng of (apparent) progesterone/ml while the total calculated contribution of known steroid compounds to this response (about 300 Pg/ml) was much

smaller. The interference seems unlikely to be due to an effect on the separation system since neither the double antibody nor the solid phase system used in these experiments is distorted by differences in the amount of plasma present in the incubation mixtures. The grossly high values obtained in the assays of unextracted plasma could be due to unidentified steroids or compounds with related structures. However interference could also be due to the non-specific inhibition of the primary antigen-antibody reaction due to competition from progesterone-binding proteins in the plasma.

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